Studies on the Anti-obesity Effects of Major Isoflavones in the Hot Water Extract of Pueraria Flowers(Dissertation_全文)

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Contents

Introduction

Obesity is a well-established risk factor for developing hypertension, diabetes, dyslipidemia, and cancers, and it causes premature death [1]. Most importantly, an increase in visceral fat area is responsible for many of the metabolic abnormalities, including impaired glucose tolerance, insulin resistance, and increased very low-density lipoprotein triglyceride (VLDL-TG) associated with abdominal obesity [2-4]. In addition, it is reported that approximately 50% of cases of obesity involving visceral fat accumulation are complicated by fatty liver [5]. Fatty liver is a reversible condition in which triglycerides accumulate in large vacuoles in hepatocytes. Severe fatty liver is occasionally accompanied by inflammation, a situation that is referred to as steatohepatitis. When inflammation and steatohepatitis occur in people who do not drink alcohol, the condition is called non-alcoholic steatohepatitis (NASH). Hence, a reduction in visceral fat has become a key therapeutic goal in the management of obesity [6].

Puerariae flower extract (PFE) is a hot water extract of the Kudzu flower (*Puerariae thomsonii*). Kudzu is a leguminous plant that grows in Japan, China, and the other countries. The Kudzu flower is frequently used in traditional Chinese medicine for counteracting symptoms associated with alcohol use [7]. Additionally, tea made from dried Kudzu flowers is widely consumed in South China [8]. The following isoflavones are found in the Kudzu flower: 6-hydroxygenistein 6,7-di-*O*-glucoside (6HGDG), tectorigenin 7-*O*-xylosylglucoside (TGXG), tectoridin, genistin, glycitin, tectorigenin, genistein, and glycitein [9]. Among these isoflavones, 6HGDG, TGXG, tectoridin and tectorigenin, which comprise nearly 90% of the isoflavones in the Kudzu flower, are present in PFE. It has been reported that soy isoflavones, such as genistein and daidzein, exert anti-obesity effects [11, 12]. Recently, Kim *et al.* demonstrated that daidzein supplementation prevented obesity and non-alcoholic fatty liver disease in an animal study [13]. In addition, Wang *et al.* confirmed that flavones derived from *Radix Puerariae* exert inhibitory effects on body weight, abdominal fat content, and lipid levels in the liver [10]. It is therefore believed that

isoflavones may have the potential to prevent obesity and fatty liver disease. However, there have been no studies on the effects of Kudzu flower on adipose tissue weight and non-alcoholic fatty liver disease.

Therefore, in this study, we aimed to investigate the effect of PFE on adipose tissue weight in mice and humans and elucidate the mechanism underlying the anti-obesity effects of PFE and its major isoflavones. In chapter 1, we have reported the anti-obesity effects of PFE and its isoflavone-rich fraction in mice fed a high-fat diet. In chapter 2, we have reported two clinical studies to clarify the effective dosage of PFE for humans. In chapter 3, we have reported the estrogenic activity and gene expression related to lipid metabolism to elucidate the mechanism of PFE.

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Chapter 1

Pueraria Flower Extract and Its Isoflavone-rich Fraction have Anti-obesity and Anti-fatty Liver Effects in High-fat Diet-fed Mice

Introduction

Obesity is a well-established risk factor for developing hypertension, diabetes, dyslipidemia, and cancers, and it causes premature death [1]. Most importantly, an increase in visceral fat area is responsible for many of the metabolic abnormalities, including impaired glucose tolerance, insulin resistance, and increased very low-density lipoprotein triglyceride (VLDL-TG) associated with abdominal obesity [2-4].

Puerariae flower extract (PFE) is a hot water extract of the flowers of Kudzu (*Puerariae thomsonii*). It contains approximately 20 percent of isoflavones as the major ingredient. Kudzu, a leguminous plant distributed in Japan, China, and other areas, has long been used in folk medicine. In particular, *Puerariae* flowers are used in Japanese and Chinese folk medicine for treating hangovers [5-7]. Niiho *et al.* confirmed that the *Puerariae lobata* flower exerts hepatoprotective effects in individuals with liver injury induced by carbon tetrachloride or a high-fat diet in animal studies [5, 7]. Recently, research of the effects of Kudzu on lipid metabolism was reported. Wang *et al.* confirmed that flavones derived from *Radix Puerariae* exert inhibitory effects on body weight, abdominal fat content, and lipid levels in the liver [8].

It has been reported that soy isoflavones, such as genistein and daidzein, exert anti-obesity effects [9, 10]. Recently, Kim *et al.* demonstrated that daidzein supplementation prevented obesity and non-alcoholic fatty liver disease in an animal study [11]. As previously described, PFE is a rich source of isoflavones, it is therefore believed that PFE may have the potential to prevent obesity and fatty liver disease. However, there have been no studies on the effects of Kudzu flower and its isoflavones on adipose tissue weight and hepatic triglyceride levels.

Here, we conducted an animal study to investigate the effects of PFE and the isoflavone-rich

fraction (ISOF) of PFE on adipose tissue weight and hepatic triglyceride levels. In addition, we analyzed fecal lipids to investigate the effect of PFE on lipid absorption.

Materials and methods

Experimental materials

Puerariae flower extract was purchased from Ohta's Isan Co. Ltd. (Ushiku city, Japan). This compound contains 7 isoflavones: tectoridin (4.70%), tectorigenin 7-*O*-xylosylglucoside (8.37%), 6-hydroxygenistein-6,7-diglucoside (3.38%), glycitin (0.17%), tectorigenin (0.83%), glycitein (0.10%), and genistein (0.06%). All isoflavone standard preparations we used were purchased from either Nagara Science Co., Ltd. (Gifu, Japan) or Tokiwa Phytochemical Co., Ltd. (Chiba, Japan).

Fractionations

The extract was dissolved in 20% MeOH and then sequentially eluted with 20%, 40%, 60%, and finally 100% MeOH for column chromatography (Cosmosil 75C18-OPN, Nacalai Tesque Inc., Kyoto, Japan). The fraction obtained from 20% MeOH was considered to be fraction 1. Next, silica gel column chromatography (solvent A, 1:1:40 [v/v) MeOH:HCOOH:$CHCl_3$; solvent B, 10:1 [v/v) MeOH:HCOOH) was performed using the 60% MeOH fraction. The solvent-A-eluted fraction and the 40% MeOH fraction were then mixed to obtain the ISOF. Fraction 2 was obtained by mixing the solvent-B-eluted fraction with the 100% MeOH fraction (Fig. 1). The fractionation yields, calculated as the dry weight of the fraction, were 27.1% for ISOF, 67.4% for fraction 1, and 3.9% for fraction 2.

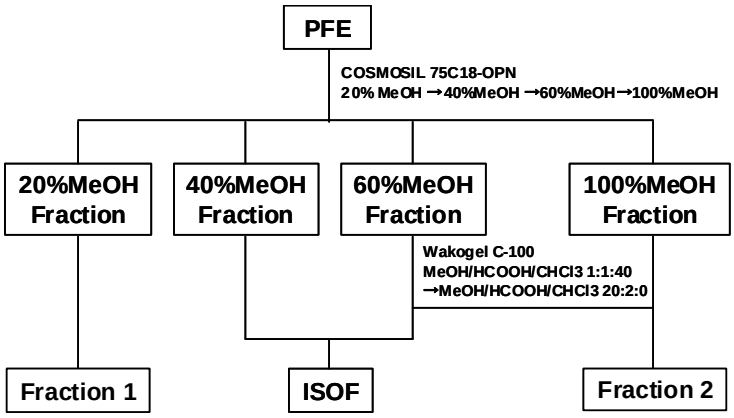

Figure 1. Fractionation flow of ISOF prepared from PFE

Quantitative isoflavone estimation

The dietary soy isoflavone-aglycone testing method (See Food Safety Notification No. 0823001, August 23, 2006 for guidelines regarding handling of specified health food including soy isoflavones) was modified and implemented to quantify the amount of isoflavones present in PFE and each of the previously described fractions. Each fraction and PFE were dissolved in 50% EtOH and analyzed with high-performance liquid chromatography using a 4.6 × 250 mm YMC-pack ODS-AM-303 column, with UV detection at 264 nm. The flow rate was 1.0 mL/min. Solvent A was CH_3CN:H_2O:CH_3COOH at a ratio of 15:85:0.1 (v/v), and solvent B was CH_3CN:H_2O:CH_3COOH at a ratio of 35:65:0.1. Quantitative isoflavone results are shown in Table 1.

Table 1. Results of quantitative isoflavone estimation in each fraction

(g/100g)

Constituent name	PFE	ISOF	Fraction1	Fraction2
6-Hydroxygenistein 6,7-di-O-glucoside	3.38	12.14	N.D.	N.D.
Glycitin	0.17	0.66	N.D.	N.D.
Tectorigenin 7-O-xylosylglucoside	8.37	30.35	N.D.	N.D.
Genistin	0.27	0.95	N.D.	N.D.
Tectoridin	4.70	16.30	N.D.	1.19
Glycitein	0.10	0.27	N.D.	N.D.
Genistein	0.06	0.16	N.D.	N.D.
Tectorigenin	0.83	2.30	N.D.	N.D.
Total	17.87	63.11	N.D.	1.19

Experimental animals and diet

All animal procedures were performed in accordance with the Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animal Science and were approved by the Ethical Committee of Toyo Shinyaku Co., Ltd. Male C57BL/6J mice were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan) at the age of 6 weeks. At 7 weeks, the mice were divided into 3 groups: (1) high-fat diet (HFD), (2) high-fat diet and given 5% PFE (HFD + PFE), and (3) high-fat diet and given PFE ISOF (HFD + ISOF). All groups were fed for 42 days. For the HFD + ISOF group, 1.355% ISOF from the fractionation yield was used. The animals were kept in an air-conditioned environment with a 12-h light cycle (lights on from 0800–2000). Mice were fed ad libitum during preparatory breeding and were on a controlled feeding regimen during the testing period. During the study, the animals were weighed every 4 days. Food intake was determined every day by subtracting the food remaining in the feed container from the total amount given the day before. The feed composition is shown in Table 2.

Table 2. Feed composition

(g/100g)

	HFD	HFD+PFE	HFD+ISOF
Casein	20.0	20.0	20.0
α-potato starch	28.2	23.2	26.845
Sucrose	13.0	13.0	13.0
Corn oil	20.0	20.0	20.0
Rard	10.0	10.0	10.0
Cellulose	4.0	4.0	4.0
Mineral Mix (AIN-76)	3.5	3.5	3.5
Vitamin Mix (AIN-76)	1.0	1.0	1.0
DL-Methionine	0.3	0.3	0.3
PFE	0.0	5.0	0.0
ISOF	0.0	0.0	1.355
Total	100.0	100.0	100.0

Analysis of fecal lipids

Beginning on day 38 (after starting the diet regime), feces were collected for at least 3 days,

from which fecal lipids were measured. The feces were dried for at least 3 days at 100°C and their weight was then measured. They were then pulverized and submitted for gross fecal lipid measurement. To determine gross fecal lipid weight, lipid was extracted using the method described by Folch *et al.* [12]

Results

Food Intake, Body Weight, Adipose Tissue Weight, and Fecal Lipid Levels

Final body weight, weight gain, and white and brown adipose tissue weights were significantly lower in the HFD + PFE group compared to the HFD group (Table 3). This suggests that PFE inhibits the fat weight gain caused by a high-fat diet. The HFD + ISOF group had similar results as the HFD + PFE group, and were not significantly different. This suggests that the active ingredient in PFE preventing weigh gain is also present in the ISOF. There were no significant differences in food intake or fecal lipid levels between the HFD + PFE and HFD + ISOF groups compared to the HFD. Because of this, the anti-obesity effects of PFE and ISOF are likely not due to differences in energy intake.

Hepatic Triglyceride Levels

Hepatic triglyceride levels in both the HFD + PFE and HFD + ISOF groups were significantly lower than those in the HFD group (Table 3).

Table 3. Food intake, body weight, adipose tissue relative weights, fecal lipid, and hepatic triglyceride in male C57BL/6J mice fed HFD, HFD+PFE, and HFD+ISOF diets for 42 day

	HFD	HFD+PFE	HFD+ISOF
Food intake, g/day	2.8±0.1	2.6±0.1	2.7±0.0
Final body weight, g	31.5±0.7 a	27.3±0.6 b	28.2±0.4 b
Body weight gain, g	7.3±0.8 a	3.3±0.3 b	3.9±0.4 b
White adipose tissue weight			
Epididymal, g/100g body weight	5.2±0.3 a	2.7±0.2 b	3.3±0.2 b
Mesentric, g/100g body weight	1.4±0.1 a	0.8±0.1 b	0.9±0.0 b
Retroperitoneal, g/100g body weight	0.4±0.0 a	0.2±0.0 b	0.2±0.0 b
Total, g/100g body weight	6.9±0.4 a	3.8±0.3 b	4.5±0.2 b
Brown adipose tissue weight, g/100g body weight	1.0±0.0 a	0.6±0.0 b	0.7±0.0 b
Fecal lipid, g/day	0.022±0.002	0.025±0.003	0.023±0.004
Hepatic triglyceride, mg/g wet tissue	46.7±3.7 a	31.5±2.5 b	26.2±1.5 b

Note: The data represent the mean ± SEM values (n = 7-8). Different symbols represent $p<0.05$ as compared with the other groups

Discussion

It has been reported that soy isoflavones, such as genistein and daidzein, exert anti-obesity effects [9, 10]. Recently, Kim *et al.* demonstrated that daidzein supplementation prevented obesity and non-alcoholic fatty liver disease in an animal study [11]. It is therefore believed that isoflavones may have the potential to prevent obesity and fatty liver disease.

The present study indicates that dietary PFE and its isoflavone-rich fraction both produce anti-obesity effects in high-fat diet-fed mice. There were no significant differences between the HFD + PFE and HFD + ISOF groups in final body weight, weight gain, or white adipose tissue weight. Similarly, for these groups there were no significant differences in the amount of isoflavone intake (23.4 ± 1.0 mg/day for HFD + PFE and 23.4 ± 0.3 mg/day for HFD + ISOF), as calculated from the isoflavone content of PFE and ISOF (Table 1), dietary composition (Table 2), and amount of food intake (Table 3). Accordingly, our findings suggest that PFE has anti-obesity effect and the active ingredients responsible for the PFE anti-obesity effect are indeed isoflavones.

Within PFE there are 4 major types of isoflavones: 6-hydroxygenistein 6,7-di-*O*-glucoside, tectorigenin 7-*O*-xylosylglucoside, tectoridin, and tectorigenin (Table 1). When cultured with

enteric bacteria, tectoridin and tectorigenin-7-*O*-xylosylglucoside are metabolized into tectorigenin, and 6-hydroxygenistein 6,7-di-*O*-glucoside is metabolized into 6-hydroxygenistein (an aglycone isoflavone) [13, 14]. Tectorigenin is detected in the urine after oral administration of PFE in human subjects (unpublished data). Consequently, the major isoflavones of PFE are likely absorbed into the bloodstream in tectorigenin form. Our recent unpublished data show that tectorigenin suppresses hepatic lipid accumulation in HepG2 cells. In the present study, hepatic triglyceride levels of both the HFD + PFE and HFD + ISOF groups were significantly lower than that in the HFD group (Table 3). This result may be caused by tectorigenin; however, future studies are required to determine the underlying mechanisms responsible for this.

In summary, we conducted an animal study to investigate the effect of PFE and ISOF on adipose tissue weight, hepatic triglyceride, and fecal lipid. As a result, the active ingredient causing the anti-obesity effect of PFE is thought to be an isoflavone. In addition, PFE and ISOF did not affect lipid absorption.

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Chapter 2

Clinical Studies on the Anti-obesity Effects of *Pueraria* Flower Extract on Obese Humans

Section 1

Preliminary Research for the Anti-obesity Effect of *Pueraria* Flower Extract in Humans

Introduction

Obesity is a major public health issue, as it is closely related to mortality. Obesity is caused by a variety of factors, one of which is an imbalance between energy intake and expenditure. Therefore, it is important for the prevention of obesity to suppress energy intake and increase its expenditure. Diet therapy is generally advocated for suppressing energy intake, while exercise is recommended for ensuring adequate energy expenditure. In addition, the use of a dietary supplement that can reduce energy intake by inhibiting the absorption of sugar and fat and/or elevate energy metabolism in the liver and muscles via β-oxidation is recommended.

Pueraria Flower Extract (PFE) is an extract from flowers of Kudzu (*Puerariae thomsonii*). Kudzu, a leguminous plant distributed in Japan, China, and other areas, has long been used in folk medicine. In particular, the *Pueraiae* flower is used in Japanese and Chinese folk medicine for curing hangovers [1-3].

Niiho *et al* confirmed that the consumption of the *Puerariae lobata* flower reduces glutamic oxaloacetic transaminase (GOT) or glutamic pyruvic transaminase (GPT) levels in individuals with liver injury due to carbon tetrachloride (CCl_4) or a high-fat diet in animal study [1, 2]. In East Asia, this flower has also recently been used in herbal medicines for the management of menopausal symptoms [4, 5].

Puerariae thomsonii is known to contain 7 isoflavones (4 isoflavone glucosides: tectoridin,

tectorigenin 7-*O*-xylosylglucoside, 6-hydroxygenistein-6,7-diglucoside, and glycitin; and 3 aglycones: tectorigenin, glycitein, and genistein) [6] . Our previous studies have shown that PFE and its isoflavone rich fraction supplementation exert anti-obesity and anti-fatty liver effects in high-fat diet-fed mice. However, the impact of PFE on body fat in humans is not known. This clinical study was preliminary conducted on eighty mildly obese subjects to view the body fat reducing action of PFE in humans.

Materials and methods

Design and Subjects

We had drew up a clinical protocol for this study. Then this study had been approved by Institutional Review Board of Kurume clinical pharmacology clinic (July 28, 2005) before its launch. Furthermore this study was conducted in accordance with the Helsinki Declaration, and informed consent was obtained from all subjects. We entrusted all affairs of this study to Iberica Holdings Co., Ltd. (Fukuoka city, Japan). They carried it out at Kurume clinical pharmacology clinic (Kurume city, Japan).

Volunteers who live in around Kurume city had a questionnaire related to previous disease and administration of drugs, blood tests, body checks and a medical interview by a doctor. Subjects aged between 20 and 65 years were recruited for the study, according to fixed inclusion and exclusion criteria. The inclusion criteria pertained to the following prospective subjects: (1) Those having a body mass index (BMI) value of 23–30, or a waist circumference larger than 85 cm for males or 90 cm for females; (2) Those who had visited a hospital for treatment and provided informed consent for study enrolment; (3) Those who were deemed to be suitable for the study by a doctor; (4) Those who had been certified as healthy by a doctor, during a prior clinical inspection, and (5) Those who were not under any drug treatment.

The exclusion criteria pertained to the following prospective subjects: (1) Those using functional foods, cosmetics, or instruments that may influence lipid metabolism (e.g.

hypolipidaemic compounds and antihypertensive agents); (2) Those with liver dysfunction; (3) Pregnant women, or those suspected to be pregnant; (4) Those who were considered unsuitable for the study due to an illness or the possibility of developing serious side effects; and, (5) Those who were deemed to be inappropriate subjects by a doctor.

Test food

Tablets containing PFE (Ohta's Isan Co. Ltd., Ushiku city, Japan), reduced palatinose, cellulose, fatty acid esters of sucrose, and silicon dioxide were used as the test food. The placebo food consisted of tablets containing caramel as a dye, instead of PFE. Table 1 shows analysis value of nutrient composition. The test food and the placebo food were distributed with plain aluminium packets sealed individually containing 1.0g per packet. The subjects in each group were required to consume the allotted tablet once per day.

Table 1 Analysis value of nutrient composition for Test food (1.0g per packet)

	Test food (containing 100mgPFE)	Test food (containing 200mgPFE)	Test food (containing 300mgPFE)	Placebo food
Energy[1)] (kcal)	3.94	3.88	3.84	3.96
Moisture (g)	0.036	0.039	0.040	0.037
Protein[2)] (g)	0.019	0.039	0.059	0.001
Fat (g)	0.033	0.036	0.040	0.031
Carbohydrate (g)	0.891	0.851	0.812	0.918

1) Calorie conversion factor : Protein 4, Fat 9, Carbohydrate 4

2) Nitrogen・protein conversion factor : 6.25

Experimental design

Double-blind placebo controlled study was conducted in this case. Figure 1 shows the test schedule. Following a 2-week pre-observation period (i.e., at "week 0"), haematological, urine, and physical examinations and blood biochemical analyses were conducted at the hospital. Further, 80

subjects with mild obesity were randomly divided into 4 groups using block randomization. Subjects in groups I, II, and III were required to ingest 100, 200, and 300 mg of PFE, respectively, while those in group IV were required to ingest the placebo food once per day for 8 weeks. Subjects were restricted to take medicines and supplements as a general rule and had dietary instruction to prevent from rapid change of calories consumed during the intake of PFE. We distributed pedometers to the subjects to instruct not to make rapid change to their amount of exercise. Regarding other daily habit, we instructed to have a life as usual. Subjects were also asked to keep the daily record of test food consumption, rational symptoms, all foods consumed including articles of taste, amount of exercise and intake of prohibited medicines or supplements.

Examinations were conducted at 4 and 8 weeks after week 0 (i.e., during week 4 and week 8). Blood biochemical analyses and physical examinations were conducted at week 4 and additional haematological and urine examinations and blood biochemical analyses were conducted at week 8. Furthermore, subjective symptoms were recorded 2 weeks after the study period.

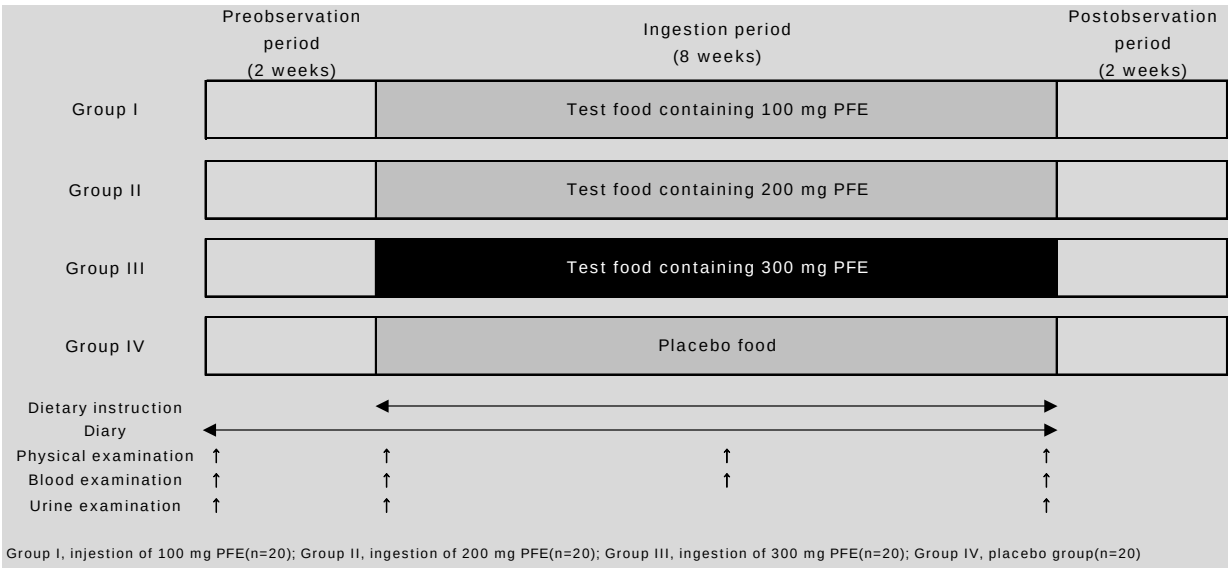


Figure 1 Test schedule

Dietary instruction

The subjects were instructed to limit their total energy intake to under 2650 kcal/day in the

case of males, and 2300 kcal/day in the case of females, and also instructed not to reduce food intake substantially basing on the Dietary Reference Intakes for Japanese, 2005. The details of their meal intake are provided below.

Breakfast: Bread or rice, a side dish, and milk;

Lunch: Nutritious foods for which the total energy intake could be measured and the nutrients classified;

Dinner: A home-delivered meal (main dish, 300 kcal; side dish, 320 kcal);

With regard to alcohol consumption, the subjects were permitted less than 1 large bottle of beer or the equivalent. Alcohol consumption was forbidden on the day before a blood examination or medical interview.

Haematological examinations and biochemical examinations of blood

Haematological examinations were conducted at weeks 0 and 8. Blood biochemical analyses were conducted at weeks 0, 4, and 8.

The parameters assessed in the haematological examinations were the white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (Hb) level, and haematocrit (Ht) value. Those assessed in the blood biochemical analyses performed at week 4 were the triglyceride, total cholesterol (T-Cho), low-density-lipoprotein cholesterol (LDL-Cho), high-density-lipoprotein cholesterol (HDL-Cho), remnant-lipoprotein cholesterol (RLP-Cho), leptin, fasting blood glucose (FBG), and insulin levels. In addition, the parameters assessed in the blood biochemical analyses performed at week 8 were the total proteins, and the GOT, GPT, γ-glutamyl transpeptidase (γ-GTP), albumin, creatinine, total bilirubin (TB), blood urea nitrogen (BUN), uric acid, creatine kinase (CPK), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), triglyceride, T-Cho, LDL-Cho, HDL-Cho, RLP-Cho, leptin, FBG, insulin, and haemoglobin A1c (HbA1c) levels.

Urine examinations

Urine examinations were conducted at weeks 0 and 8. The parameters assessed were the urine sugar and protein content and the presence of blood in the urine.

Rational symptoms

Subjective symptoms, which occurred during the study, were tallied. Symptoms, which confirmed a relation to the test food by a doctor, were also tallied.

Physical examinations

Physical examinations were conducted at weeks 0 and 8, and the parameters assessed were height (data not shown), body weight, BMI, waist circumference, hip circumference, total fat area, visceral fat area, subcutaneous fat area, systolic blood pressure, and diastolic blood pressure.

The hip circumference was measured using a calibrated tape. The waist circumference, total fat area, visceral fat area, and subcutaneous fat area were measured by performing computerized tomography (CT) while the subjects held their breath following expiration. The CT scan data were analysed using the Fat scan software (East Japan Institute of Technology Co. Ltd., Hitachi city, Japan) [7].

Statistical analysis

We performed a repeated-measures one-way analysis of variance (ANOVA) to investigate the changes over the time course. When statistically significant differences were detected, a post-hoc pairwise comparison across treatments was performed using Fisher's probability of least significant difference (PLSD) test for multiple comparisons. For the comparison among groups, analyses were performed by Tukey-Kramer test on all test items. However, this study is preliminary. Therefore, distinctions of weight index relating to body fat, BMI, waist circumference, hip circumference, total fat area, subcutaneous fat area and visceral fat area between the group consumed test food (100mg,

200mg, 300mg) and the group consumed placebo food were analysed by unpaired t-test.

All statistical analyses were performed using Statview ver. 5.0 (SAS Institute Japan Ltd., Tokyo, Japan), and significance was set at $p < 0.05$.

RESULTS

Exclusion from the study

One subject in group II withdrew his consent for participation and was excluded from the study; however, this was not due to any problem with the test food.

Subjects who had not eaten home-delivered meals for more than 10% of all meals were excluded from the study. The number of subjects was 18, 15, 18, and 18 for groups I, II, III, and IV, respectively. Subject characteristics are listed in Table 2.

Table 2 Subject characteristics

	Group I (n = 18)	Group II (n = 15)	Group III (n = 18)	Group IV (n = 18)
Number	Male: 16 Female: 2	Male: 12 Female: 3	Male: 15 Female: 3	Male: 15 Female: 3
Age (years)	40.3 ± 7.9	37.9 ± 9.1	39.3 ± 12.4	36.7 ± 9.4
Body weight (kg)	75.2 ± 9.1	74.2 ± 11.8	72.0 ± 9.4	76.1 ± 8.6
BMI (kg/m^2)	26.7 ± 2.6	26.4 ± 3.2	25.5 ± 2.9	26.5 ± 2.5
Waist circumference (cm)	95.2 ± 8.5	95.2 ± 10.1	93.3 ± 7.6	96.8 ± 8.4
Hip circumference (cm)	99.9 ± 5.4	98.6 ± 5.7	99.8 ± 4.4	101.4 ± 4.2
Total fat area (cm^2)	343.0 ± 82.4	323.0 ± 126.4	306.6 ± 92.0	331.4 ± 85.4
Visceral fat area (cm^2)	128.5 ± 38.1	133.8 ± 115.6	111.3 ± 44.2	122.8 ± 53.5
Subcutaneous fat area (cm^2)	214.5 ± 65.1	189.2 ± 52.4	195.3 ± 65.2	208.6 ± 59.4
Systolic blood pressure (mmHg)	128.9 ± 11.4	128.5 ± 17.3	120.1 ± 9.4	126.1 ± 15.4
Diastolic blood pressure (mmHg)	82.2 ± 7.3	80.3 ± 15.2	74.9 ± 9.6	80.8 ± 12.6

See Figure 1 for Groups.

Values are means ± SD.

Haematological examinations and blood biochemical analyses

The results of the haematological examinations are shown on Table 3. At week 8, the WBC count in group III and the Ht value in group I had changed significantly from the initial values. However, the WBC count remained within the normal range. Although the average Ht values recorded for group I at week 8 exceeded the normal values for females, the values recorded for individual female subjects in this group were all within the normal range. Thus, no abnormalities were noted during the haematological examinations (RBC, WBC, Ht, and Hb).

The results of the blood biochemical analyses are shown in Table 4. Although the T-Cho and HDL-Cho levels in all the groups had reduced significantly at week 4 compared to week 0, they returned to their original values at week 8. The LDL-Cho levels reduced significantly in groups I and III at week 4, but returned to their original normal valves at week 8. Although the LDL-Cho

levels recorded for group III exceeded the normal range at week 8, these levels were higher than normal, even at week 0. Therefore, the changes in these levels were not considered abnormal. The leptin levels were significantly lower in group III at both of weeks 4 and 8, as compared to week 0. Group I significantly showed low value at weeks 8, as compared to week 0. Since leptin is secreted from fat tissue, this result may be attributable to a reduction in body fat. The TB level in group II; GOT and GPT levels in groups I, II, and III; uric acid levels in groups I and III; and the ALP level in group III were all significantly different from the corresponding values at week 0, but remained within the normal range. Although the average γ-GTP values of groups I and III were significantly lower than the week 0 values, they exceeded the normal range for females. Nevertheless, the values recorded for individual female subjects in these 2 groups were all within the normal range. No significant changes were noted in the other parameters for any of the groups.

Table 3 Hematological examination

	Group	number	0 week	8 week	
White blood cell count ($\times 10^3/\mu L$)	I	18	6.644 ± 1.641	6.600 ± 1.721	
	II	15	6.693 ± 2.045	6.513 ± 1.904	
	III	18	6.967 ± 1.304	6.439 ± 1.501	*
	IV	18	6.576 ± 1.196	6.694 ± 1.555	
Red blood cell count ($\times 10^6/\mu L$)	I	18	4.99 ± 0.38	5.05 ± 0.38	
	II	15	4.85 ± 0.38	4.94 ± 0.34	
	III	18	5.00 ± 0.39	5.00 ± 0.39	
	IV	18	4.92 ± 0.44	4.93 ± 0.47	
Hemoglobin level (g/dL)	I	18	15.24 ± 1.23	15.22 ± 1.26	
	II	15	15.01 ± 1.49	15.14 ± 1.32	
	III	18	15.16 ± 1.41	15.09 ± 1.40	
	IV	18	14.98 ± 1.22	14.83 ± 1.31	
Hematocrit (%)	I	18	45.66 ± 3.23	46.65 ± 3.38	**
	II	15	44.62 ± 3.80	45.79 ± 3.42	
	III	18	45.10 ± 3.77	45.55 ± 4.11	
	IV	18	44.87 ± 3.18	45.39 ± 3.55	

See Figure 1 for Groups.

Values are means ± SD.

* and ** indicate significantly different from the value obtained in week 0 at $p < 0.05$ and $p < 0.01$, respectively.

Table 4-1 Biochemical examination (liver and renal function)

	Group	Number	Week 0	Week 8	
Total protein (g/dL)	I	18	7.41 ± 0.36	7.38 ± 0.36	
	II	15	7.29 ± 0.25	7.25 ± 0.32	
	III	18	7.41 ± 0.25	7.31 ± 0.33	
	IV	18	7.41 ± 0.26	7.29 ± 0.32	
Albumin (g/dL)	I	18	4.57 ± 0.24	4.62 ± 0.25	
	II	15	4.50 ± 0.21	4.55 ± 0.24	
	III	18	4.59 ± 0.17	4.56 ± 0.18	
	IV	18	4.62 ± 0.22	4.61 ± 0.22	
Total bilirubin (mg/dL)	I	18	0.67 ± 0.17	0.68 ± 0.27	
	II	15	0.72 ± 0.23	0.61 ± 0.22	*
	III	18	0.72 ± 0.35	0.64 ± 0.27	
	IV	18	0.68 ± 0.23	0.65 ± 0.22	
GOT (IU/L)	I	18	26.4 ± 7.8	22.0 ± 5.3	**
	II	15	25.5 ± 13.6	19.9 ± 7.0	*
	III	18	24.0 ± 11.3	20.4 ± 7.6	*
	IV	18	24.8 ± 8.4	24.9 ± 15.9	
GPT (IU/L)	I	18	43.7 ± 24.4	35.1 ± 22.2	*
	II	15	37.3 ± 31.1	25.9 ± 16.8	*
	III	18	38.4 ± 26.5	29.2 ± 19.7	***
	IV	18	36.2 ± 24.1	37.2 ± 29.8	
ALP (IU/L)	I	18	221.1 ± 39.8	226.7 ± 50.4	
	II	15	221.2 ± 52.1	224.4 ± 55.3	
	III	18	195.4 ± 47.9	206.6 ± 50.7	*
	IV	18	213.4 ± 62.6	220.5 ± 65.3	
LDH (IU/L)	I	18	195.7 ± 34.4	186.4 ± 25.7	
	II	15	188.0 ± 33.6	186.7 ± 27.7	
	III	18	177.0 ± 28.1	170.3 ± 24.1	
	IV	18	190.5 ± 27.7	191.6 ± 26.5	
γ-GTP (IU/L)	I	18	55.6 ± 33.6	42.9 ± 21.7	**
	II	15	51.3 ± 46.4	41.3 ± 28.1	
	III	18	43.7 ± 37.7	34.4 ± 24.2	*
	IV	18	45.4 ± 32.5	48.9 ± 39.7	
BUN (mg/dL)	I	18	14.6 ± 4.3	12.7 ± 2.5	
	II	15	12.5 ± 2.2	12.1 ± 2.9	
	III	18	12.8 ± 2.1	13.3 ± 3.0	
	IV	18	13.1 ± 2.5	12.8 ± 2.5	
Creatinine (mg/dL)	I	18	0.80 ± 0.13	0.79 ± 0.13	
	II	15	0.81 ± 0.14	0.80 ± 0.14	
	III	18	0.79 ± 0.12	0.79 ± 0.11	
	IV	18	0.77 ± 0.10	0.78 ± 0.10	
Uric acid (mg/dL)	I	18	6.55 ± 1.70	5.94 ± 1.55	***
	II	15	6.21 ± 1.51	6.05 ± 1.09	
	III	18	5.98 ± 1.60	5.68 ± 1.55	*
	IV	18	6.09 ± 1.29	6.07 ± 1.52	
CPK (IU/L)	I	18	155.3 ± 81.9	156.6 ± 101.7	
	II	15	118.0 ± 52.4	118.4 ± 47.7	
	III	18	110.9 ± 43.2	104.6 ± 33.6	
	IV	18	127.4 ± 52.9	124.1 ± 743.3	

See Figure 1 for Groups.

Values are means ± SD.

*,**, and *** indicate significantly different from the value obtained in week 0 at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Table 4-2 Biochemical examination (lipid and glucose levels)

	Group		Number	Week 0	Week 4		Week 8	
Total cholesterol (mg/dL)	I		18	197.2 ± 25.1	186.8 ± 26.9	**	198.1 ± 27.7	
	II		15	202.1 ± 54.3	191.0 ± 45.0	**	202.3 ± 54.4	
	III		18	215.4 ± 29.9	194.0 ± 23.2	***	208.1 ± 29.9	
	IV		18	208.6 ± 43.7	194.1 ± 33.5	**	209.2 ± 30.8	
LDL cholesterol (mg/dL)	I		18	135.7 ± 24.1	126.2 ± 25.9	**	134.0 ± 26.5	
	II		15	139.4 ± 54.4	127.9 ± 43.9		133.8 ± 51.6	
	III		18	146.7 ± 27.1	132.0 ± 18.0	**	139.1 ± 25.5	
	IV		18	141.0 ± 34.8	131.5 ± 31.0		137.1 ± 28.5	
HDL cholesterol (mg/dL)	I		18	48.8 ± 9.4	43.6 ± 6.8	***	49.1 ± 8.1	
	II		15	52.6 ± 8.3	49.5 ± 8.7	*	52.9 ± 10.2	
	III		18	50.2 ± 10.7	47.7 ± 9.9	*	50.7 ± 11.4	
	IV		18	49.7 ± 13.1	45.8 ± 7.9	*	51.2 ± 9.9	
RLP cholesterol (mg/dL)	I		18	6.4 ± 3.2	6.6 ± 3.6		5.1 ± 2.9	
	II		15	4.9 ± 2.4	4.9 ± 2.8		4.9 ± 2.1	
	III		18	6.8 ± 5.0	5.6 ± 3.3		6.4 ± 3.9	
	IV		18	7.1 ± 3.6	5.6 ± 2.4		5.8 ± 2.1	
Triglycerides (mg/dL)	I		18	139.0 ± 71.9	160.7 ± 67.2		127.9 ± 71.3	
	II		15	104.4 ± 57.3	119.9 ± 63.8		109.6 ± 42.0	
	III		18	148.8 ± 84.6	135.4 ± 66.1		137.5 ± 62.2	
	IV		18	154.5 ± 61.2	138.2 ± 47.2		138.1 ± 44.2	
Fasting blood glucose (mg/dL)	I		18	90.3 ± 5.8	90.7 ± 7.2		91.9 ± 6.4	
	II		15	91.1 ± 6.0	91.2 ± 5.9		92.7 ± 8.9	
	III		18	89.9 ± 4.8	91.4 ± 8.9		91.1 ± 4.2	
	IV		18	90.1 ± 4.5	91.3 ± 8.0		89.7 ± 6.6	
Insulin (mU/mL)	I		18	9.2 ± 3.5	9.3 ± 4.2		9.9 ± 5.5	
	II		15	7.0 ± 3.0	7.3 ± 3.6		8.1 ± 4.0	
	III		18	8.1 ± 3.3	8.2 ± 3.6		9.1 ± 4.5	
	IV		18	11.6 ± 8.4	11.5 ± 8.7		11.1 ± 5.6	
HbA1c (%)	I		18	4.81 ± 0.22			4.76 ± 0.20	
	II		15	4.81 ± 0.20			4.83 ± 0.25	
	III		18	4.77 ± 0.35	—		4.81 ± 0.32	
	IV		18	4.82 ± 0.23			4.84 ± 0.23	
Leptin (ng/dL)	I	male	16	6.9 ± 2.4	6.0 ± 2.7		5.8 ± 3.0	*
		female	2	14.9 ± 8.1	9.9 ± 0.1		7.6 ± 0.8	
	II	male	12	7.3 ± 4.2	4.8 ± 2.0		4.9 ± 2.4	
		female	3	14.9 ± 8.1	12.8 ± 6.0		12.3 ± 4.2	
	III	male	15	6.4 ± 4.6	4.7 ± 2.9	**	4.5 ± 2.3	**
		female	3	14.2 ± 5.0	10.7 ± 2.1		10.1 ± 3.5	
	IV	male	15	7.0 ± 3.4	6.6 ± 3.9		6.2 ± 3.8	
		female	3	14.6 ± 5.7	10.6 ± 2.8		10.2 ± 2.7	

See Figure 1 for Groups.

Values are means ± SD.

*,**, and *** indicate significantly different from the value obtained in week 0 at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Urinary examinations

Sugar was not detected in the urine of any of the subjects throughout the study period. In addition, proteins were not detected in the urine of any of the subjects in groups II and III throughout the study period. For 1 subject belonging to group I, the status of urinary proteins was detected (±) at week 8; however, proteins were detected (+) in the urine of this subject even at week

0. In addition, in the case of 1 subject belonging to group IV, the status of urinary proteins was detected (±) at week 8; however, this group was the placebo group. Therefore, we considered that PFE consumption did not cause any of the abnormalities detected during the urine examinations.

Blood was detected (+) in the urine of 1 subject belonging to group III in week 8, but this subject exhibited bloody urine throughout the study period. In group II, the status of blood in the urine was assessed as (2+) in 1 subject, (+) in 1 subject, and (±) in 1 subject at week 8. In the case of the subject for whom the status was found to be (2+), the blood in the urine was confirmed to be related to menstruation, according to the recordings in a diary that had been maintained for this patient. With regard to the subject for whom the status was assessed as (+), a status ranging from (±) to (+) was noted throughout the study period. Further, in the case of the subject for whom the status of blood in the urine was assessed as ±, a similar status had been noted during prior examinations. Thus, our observations may have been due to an inherent characteristic of this subject.

In 1 subject belonging to group IV, the status of blood in the urine was found to be (2+) at week 8, which was identical to the status found at week 0. Therefore, we consider that our findings may be attributed to the inherent characteristics of this subject.

Rational symptoms

The findings with regard to the rational symptoms are shown in Table 5. Various subjective symptoms occurred in all groups. However, a doctor judged there were no relations between those symptoms and the test food.

Table 5 Number of occurrence of Rational symptoms

	Group I	Group II	Group III	Group IV
Cold	79 (0)	13 (0)	14 (0)	25 (0)
Vomiting	0 (0)	1 (0)	2 (0)	0 (0)
Skin eruption	0 (0)	0 (0)	0 (0)	0 (0)
Menstruation	2 (0)	4 (0)	21 (0)	20 (0)
Abdominal discomfort	0 (0)	0 (0)	0 (0)	2 (0)
Gastric pain	2 (0)	1 (0)	0 (0)	1 (0)
Abdominal pain	0 (0)	0 (0)	1 (0)	3 (0)
Abdominal tension	2 (0)	0 (0)	1 (0)	5 (0)
Constipation	0 (0)	0 (0)	0 (0)	9 (0)
Diarrhoea	25 (0)	16 (0)	20 (0)	36 (0)

Total sum of rational symptoms (The number of symptoms confirmed a relation to the test food by a doctor)

See Figure 1 for Groups.

Physical examination

Here, we describe the rates of change in the parameters that were assessed during the physical examinations. To evaluate the effects of PFE consumption on body fat, we analysed 58 subjects with BMI values > 24, who were defined as obese according to the criteria calculated by the analysis of covariance (ANCOVA) [8]. We analysed test items of physical examination on only male subjects because there were few female subjects. Thus, 14, 11, 11, and 14 subjects in groups I, II, III, and IV, respectively, were assessed as obese. The characteristics of these subjects are shown in Table 6, and energy intake and amount of exercise received during PFE intake are shown in Table 7. There were no significant differences among the groups with regard to these parameters.

The results of the physical examinations are shown in Table 8. At weeks 4 and 8, the BMI and body weight were significantly reduced in group III only, and not in any of the other groups. The total fat area and subcutaneous fat area were significantly reduced in both groups II and III. At week 4, the hip circumference was significantly reduced in groups I, II, and III. The visceral fat area was not significantly different from that recorded at week 0 in any of the groups. As a result of comparison among groups by Tukey – Kramer test, no significant differences were noted among the groups for any of the abovementioned parameters.

The result of comparison among groups by unpaired t-test was appeared in Fig2. Regarding weight, BMI and total fat area, group III exhibited significantly lower value than group IV (Weight data is not shown).

Table 6 Subject Backgrounds (BMI > 24) (Male)

	Group I (male) (n = 14)	Group II (male) (n = 11)	Group III (male) (n = 11)	Group IV (male) (n = 14)
Age (years)	40.3 ± 8.6	37.1 ± 8.0	37.8 ± 11.1	34.6 ± 9.0
Body weight (kg)	78.2 ± 7.9	78.4 ± 10.2	76.6 ± 8.5	78.5 ± 8.2
BMI (kg/m^2)	27.5 ± 2.4	27.1 ± 3.4	26.5 ± 3.2	26.8 ± 2.7
Waist circumference (cm)	97.2 ± 8.6	96.1 ± 11.5	97.3 ± 6.2	97.9 ± 8.5
Hip circumference (cm)	101.2 ± 5.3	99.4 ± 5.8	102.0 ± 3.9	102.2 ± 4.3
Total fat area (cm^2)	360.6 ± 83.6	330.6 ± 145.9	349.5 ± 80.2	335.5 ± 86.9
Visceral fat area (cm^2)	138.3 ± 37.1	151.3 ± 130.5	130.2 ± 40.5	131.5 ± 56.4
Subcutaneous fat area (cm^2)	222.4 ± 68.0	179.2 ± 39.1	219.4 ± 69.3	204.0 ± 54.2
Systolic blood pressure (mmHg)	130.0 ± 11.1	134.8 ± 12.2	118.5 ± 10.0	127.6 ± 16.8
Diastolic blood pressure (mmHg)	81.6 ± 8.0	84.5 ± 11.1	75.5 ± 10.4	81.1 ± 14.0

See Figure 1 for Groups.
Values are means ± SD

Table 7 Energy intake and amount of exercise (BMI > 24)(Male)

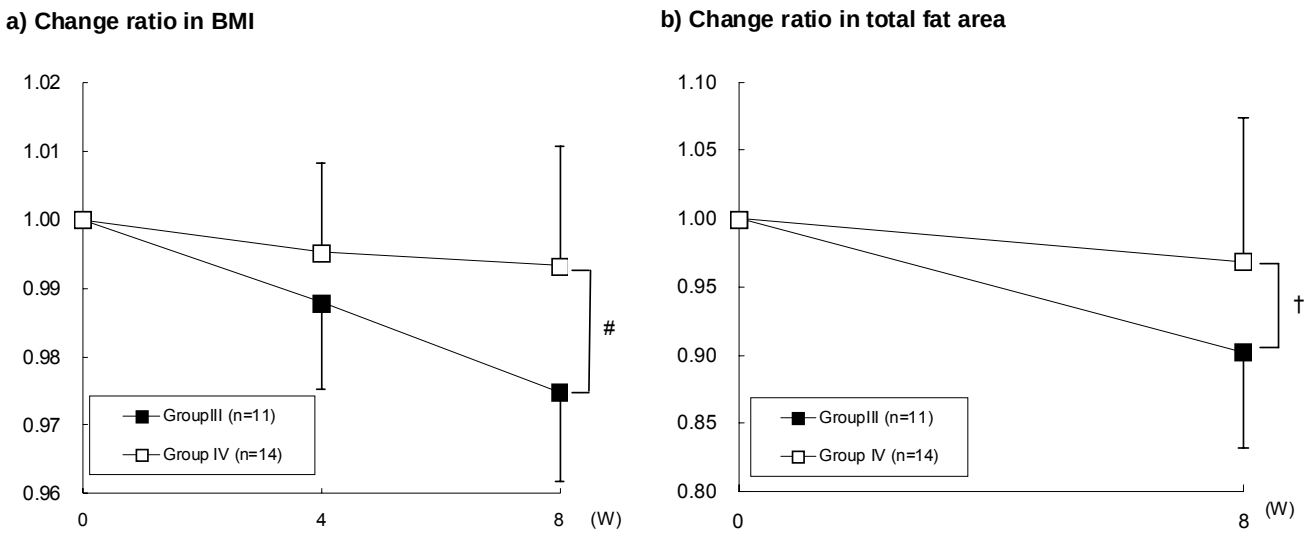
	Group I (male) (n=14)	Group II (male) (n=11)	Group III (male) (n=11)	Group IV (male) (n=14)
Total energy intake (kcal/day)	2132.5 ± 268.4	2186.3 ± 279.5	2086.2 ± 207.0	2065.1 ± 209.7
Number of steps (steps/day)	5436.2 ± 2346.5	7112.0 ± 2531.6	6126.7 ± 1635.4	6832.3 ± 2888.6

See Figure 1 for Groups.
Values are means ± SD

Table 8 Change ratio of physical examination (BMI>24) (Male)

	Group	Number	Week 0		Week 4		Week 8	
Body weight (kg)	I	14	1.000 ± 0.000		0.992 ± 0.019		0.987 ± 0.027	
	II	11	1.000 ± 0.000		0.998 ± 0.020		0.991 ± 0.023	
	III	11	1.000 ± 0.000		0.989 ± 0.013	**	0.975 ± 0.013	***
	IV	14	1.000 ± 0.000		0.995 ± 0.013		0.993 ± 0.018	
BMI (kg/m^2)	I	14	1.000 ± 0.000		0.992 ± 0.020		0.987 ± 0.027	
	II	11	1.000 ± 0.000		0.998 ± 0.021		0.992 ± 0.023	
	III	11	1.000 ± 0.000		0.988 ± 0.013	**	0.975 ± 0.013	***
	IV	14	1.000 ± 0.000		0.995 ± 0.013		0.993 ± 0.017	
Waist circumference (cm)	I	14	1.000 ± 0.000				0.996 ± 0.024	
	II	11	1.000 ± 0.000		—		0.993 ± 0.030	
	III	11	1.000 ± 0.000				0.976 ± 0.021	
	IV	14	1.000 ± 0.000				0.990 ± 0.037	
Hip circumference (cm)	I	14	1.000 ± 0.000		0.981 ± 0.029	**	0.996 ± 0.023	
	II	11	1.000 ± 0.000		0.984 ± 0.021	**	0.999 ± 0.011	
	III	11	1.000 ± 0.000		0.971 ± 0.036	**	0.984 ± 0.036	
	IV	14	1.000 ± 0.000		0.989 ± 0.021		1.007 ± 0.033	
Total fat area (cm^2)	I	14	1.000 ± 0.000				0.971 ± 0.082	
	II	11	1.000 ± 0.000		—		0.951 ± 0.060	*
	III	11	1.000 ± 0.000				0.902 ± 0.070	**
	IV	14	1.000 ± 0.000				0.968 ± 0.105	
Subcutaneous fat area (cm^2)	I	14	1.000 ± 0.000				0.979 ± 0.131	
	II	11	1.000 ± 0.000		—		0.928 ± 0.078	*
	III	11	1.000 ± 0.000				0.886 ± 0.065	***
	IV	14	1.000 ± 0.000				0.963 ± 0.122	
Visceral fat area (cm^2)	I	14	1.000 ± 0.000				0.974 ± 0.166	
	II	11	1.000 ± 0.000		—		0.958 ± 0.162	
	III	11	1.000 ± 0.000				0.950 ± 0.140	
	IV	14	1.000 ± 0.000				1.006 ± 0.211	
Systolic blood pressure (mmHg)	I	14	1.000 ± 0.000		0.949 ± 0.063		0.966 ± 0.084	
	II	11	1.000 ± 0.000		0.934 ± 0.056	**	0.921 ± 0.066	***
	III	11	1.000 ± 0.000		0.948 ± 0.082		0.965 ± 0.062	
	IV	14	1.000 ± 0.000		0.976 ± 0.051		0.961 ± 0.086	
Diastolic blood pressure (mmHg)	I	14	1.000 ± 0.000		0.970 ± 0.115		0.986 ± 0.133	
	II	11	1.000 ± 0.000		0.982 ± 0.041		0.936 ± 0.078	**
	III	11	1.000 ± 0.000		0.898 ± 0.106	**	0.901 ± 0.103	**
	IV	14	1.000 ± 0.000		0.952 ± 0.142		0.930 ± 0.140	

See Figure 1 for Groups.
Values are means ± SD
*,**,and *** indicate significantly different from the value obtained in week 0 at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.



Values are means ± SD
indicate significantly different from the value obtained in Group III and Group IV at $p < 0.01$. (unpaired t-test)
† indicate significant tencdency in Group III and Group IV at $p < 0.10$. (unpaired t-test)

Figure 2 Change ratio in BMI and total fat area（Male）

（Result of comparison between group III and group IV）

Discussion

In Japan, the number of obese persons with BMI values from 25–30 has increased by 2- to 4-fold over the last 30 years. Based on BMI values, 18.9% of the female population and 21.4% of the male population are currently considered as preobese. This may be due to the westernization of the food culture in Japan, which has increased the fat intake to twice that in the 1960s.

Obesity is the cause of many adult diseases associated with lifestyle habits, including diabetes, hyperlipidaemia, and hypertension. The type-1 plasminogen activator inhibitor (PAI-1) and agents that promote thrombus formation are excessively secreted by the cells of visceral fat tissue. This results in adiponectin hyposecretion, and this condition in turn induces insulin resistance, abnormalities in glucose metabolism, hypertension, and arteriosclerosis. Therefore, visceral adiposity is considered a dangerous condition and is termed the ‘metabolic syndrome’. To prevent its development, it is important to improve the status of obesity.

It is well known that catechin [9-11], mannooligosaccharides [12, 13], and coffee polyphenols

[14] reduce body fat; however, it is unclear whether these ingredients improve liver function. The incidence of nonalcoholic steatohepatitis (NASH) is known to be strongly associated with Obesity. Therefore, the consumption of ingredients that have effects in both reducing body fat and improving liver function is effective for the prevention of this condition.

The Puerariae flower is used in Japanese and Chinese folk medicine for curing hangovers [1-3]. Additionally, Niiho *et al* confirmed that its consumption reduces GOT or GPT levels in individuals with liver injury induced by CCl_4 or a high-fat diet in animal study [2, 3]. Further, Lee *et al* have reported the hepatoprotective effects of tectoridin and tectorigenin, which are isoflavone constituents of Puerariae flos [15]. Therefore, it is considered that the consumption of the Puerariae flos may improve liver function. Since the ability of PFE to reduce body fat *in vivo* has been confirmed, it is expected to be efficient for improving the status of both body fat and liver function.

In this study, we preliminary investigated the effects of PFE on body fat in humans. Among those subjects with BMI values of more than 24, significant reductions were noted in BMI and body weight only in group III and in the total fat area and the subcutaneous fat area in groups II and III (Table8). Although the visceral fat area was not significantly reduced in any of the groups, group III exhibited the highest reduction rate. No significant difference was confirmed on a comparison between placebo group and PFE group by Tukey-Kramer test. On the other hand, BMI value of male subjects in group III was significantly low compared with male subjects of group IV by unpaired t-test (Figure2). Although muscle mass was estimated, significant differences were not confirmed in any groups by impedance method (Data not shown). In addition, significant differences were not confirmed on creatinine and red blood cell count that related to amount of water within the body. Therefore, BMI reduction may reflect the reduction of fat area. In fact, value of total fat area of male subjects in group III tended to be reduced compared with male subjects of group IV by unpaired t-test ($p<0.10$).

GOT, GPT and γ-GTP as hepatic function markers were shown significantly low value on group I through group III at 8 weeks (Table4-1). As described above, hepatoprotective effects of

Pueraria flos has been reported with the perception from *in vivo* [2, 3]. Therefore a possibility is considered that reduction of these makers may be attributable to hepatoprotective effect. This is very interesting.

In this study, we conducted the double-blind placebo controlled study to examine whether PFE has anti-obesity effect or not preliminary. As a result, BMI value of male subjects in 300mg/day group was significantly low compared with male subjects of placebo group. Consequently, PFE intake at 300mg/day seems to have the possibility of anti-obesity effect on male. Since most subjects were male in this study, we could not confirm the actions on body fat of female. We need to confirm anti-obesity effect of PFE on female and the optimum dose for administration by a larger scale study. For the study, enough quantity of sample size should be ensured and equal numbers of male and female are required as subjects.

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Section 2

Effect of *Pueraria* Flower Extract on the Visceral Fat Area in Obese Humans (confirmatory study)

Introduction

Obesity is a well-established risk factor for developing hypertension, diabetes, dyslipidemia, and cancers, and it causes premature death [1]. Most importantly, an increase in visceral fat area is responsible for many of the metabolic abnormalities, including impaired glucose tolerance, insulin resistance, and increased very low-density lipoprotein triglycerides (VLDL-TG) associated with abdominal obesity [2-4]. Hence, a reduction in visceral fat has become a key therapeutic goal in the management of obesity [5].

Kudzu belongs to the Fabaceae family, Papollonoideae subfamily, Phaseoleae tribe, and Pueraria genus. It is known as kudzu vine, kudsu, wa yaka, and nepalem [6, 7]. It is a climbing, semi-woody, perennial vine with hairy, rusty brown stems. The flower is pea-like and is colored pink to purple with yellow center. It is highly fragrant with a sweet grape-like scent and is borne in long hanging panicles at nodes on the stem [6]. The kudzu flower is also known to be a rich source of isoflavones [7]. Recent studies have found that methanol and water extracts of the kudzu flower possess hypolipidiemic, hypoglycemic, anti-oxidant, and hepatoprotective properties *in vivo* and *in vitro* [7-11].

Previously, we investigated the effect of *Pueraria thomsonii* flower extract (PFE) on body weight in humans. The results indicated the possibility that oral intake of 300 mg of PFE reduces both body weight and abdominal fat in the mildly obese [12], but this preliminary study was conducted only on males. In the present study, we performed a long-term clinical study over 12 weeks in order to investigate the BMI reduction and visceral fat area decrease effects of PFE oral intake in obese males and females. In addition, we checked the subjects' blood biochemical parameters to observe other effects of PFE intake, if any.

Materials and Methods

Subjects

This study received the approval of the Institutional Review Board of C'est Lavie Shimbashi Clinic, Shinkokai Medical Corporation (Tokyo) in accordance with the ethical standards established in the Helsinki declaration, and informed consent was obtained from all subjects. All of the business side was entrusted to KSO Corporation (Tokyo), which carried out the study at C'est Lavie Shimbashi Clinic.

Candidate subjects were male and female volunteers aged 20 to 65 years, recruited by advertisement. A preliminary physical examination (the screening) was performed on all candidates. The study enrolled 90 candidates as test subjects, all of whom had a BMI over 25.0 kg/m^2, who did not fall under any of the following exclusion criteria:

1. Are taking drugs that might affect obesity, hyperlipidemia, or lipid metabolism;
2. Cannot stop taking supplements or functional foods that might affect obesity, hyperlipidemia, or lipid metabolism;
3. Have implanted metal in the abdominal area, as detected by computerized tomography (CT);
4. Have serious complications or have contracted a disease that requires urgent remedy;
5. Have been diagnosed with familial hyperlipidemia;
6. Have drug or alcohol dependency in the history of a present disease or medical history;
7. Are in pregnancy or lactation, or have plans to become pregnant during the study;
8. Are participating in other clinical studies, taking drugs, or applying cosmetics or drugs to the skin;
9. Are judged to be unsuitable test subjects by a physician.

The subjects were randomly categorized into three groups with equal distributions in terms of gender and BMI by a controller who was not directly involved in the trials.

Test foods

PFE, a hot-water extract of *Pueraria thomsonii* dry flowers, was purchased from Ohta's Isan (Ushiku, Japan). It contains seven isoflavones (four isoflavone glucosides, tectoridin (4.65%),

tectorigenin 7-*O*-xylosylglucoside (8.48%), 6-hydroxygenistein-6,7-diglucoside (3.53%), and glycitin (0.13%), and three aglycones, tectorigenin (0.88%), glycitein (0.07%), and genistein (0.06%)). All isoflavone standards for quantification were purchased from Nagara Science (Gifu, Japan) and Tokiwa Phytochemical(Chiba, Japan). We prepared tablets as test foods, containing 300 mg PFE, 200 mg PFE, and no PFE (placebo) per set of 12 tablets. The tablets also contained reduced palatinose, cellulose, fatty acid esters of sucrose, silicon dioxide, and caramel dye to render the tablet types indistinguishable. The nutritional compositions of the test foods are shown in Table 1.

Table1. Composition of Test Foods (per set of 12 tablets)

	Test food (containing 300 mg of PFE)	Test food (containing 200 of mg PFE)	Placebo (containing no PFE)
Energy (kcal)[1)]	11.8	11.9	11.8
Water (g)	0.090	0.087	0.090
Ptotein (g)[2)]	0.057	0.039	< 0.003
Lipid (g)	0.105	0.111	0.096
Ash (g)	0.093	0.078	0.072
Carbohydrate (g)	2.66	2.69	2.74

[1)] Calorie conversion factor: protein 4, fat 9, carbohydrate 4
[2)] Nitrogen protein conversion factor: 6.25

Study design

A double-blind, placebo-controlled parallel group study was conducted over 18 weeks, consisting of a 2-week pre-observation period (–2 w to 0 w), a 12-week test period with consumption of test foods (0 w to 12 w), and a 4-week post-observation period without test-food consumption (12 w to 16 w). The test subjects took each test food (12 tablets) 1 time per day during the test period. A physician conducted interviews, physical examinations, and blood sampling at each examination, including the pre- and post-observation periods (see below).

Examination items

The general physical examinations (except for total, visceral, and subcutaneous fat area) and biochemical blood tests were done at –2 w (for screening), 0 w (just before the test period), 4 w, 8 w,

12 w (after each test period), and 16 w (after the post-observation period). Total, visceral, and subcutaneous fat areas were detected at 0 w, 8 w, and 12 w *via* CT scan. All the test subjects were instructed to avoid raw fruits and vegetables, as well as sparkling drink intake the day before CT was performed (0 w, 8 w, and 12 w). In addition, they were prohibited from engaging in alcohol consumption 2 d before an examination, and from dietary consumption (except for water) after 21:00 on the day before an examination.

Physical examination

Height, body weight, waist circumference, and hip circumference were measured. BMI was then calculated as the body weight divided by the height squared (kg/m^2). Total, visceral, and subcutaneous fat areas were detected by CT (ProSpeed II; GE, NY). CT scan data were analyzed using conventional software (Fat Scan ver3.0; N2SM, Tokyo).

Biochemical blood test

Blood samples were collected from the subjects after overnight fasting. Triglycerides, total cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol were assessed as parameters of lipid metabolism, and leptin and adiponectine as adipocytokines, asparatate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γGTP) as hepatopathy markers, and glucose, hemoglobin A_{1c} (HbA_{1c}), and insulin as carbohydrate metabolism markers.

Management

A uniform dinner (approximately 800 kcal) was prepared for all test subjects each day (except for Sunday) during the test period with test food consumption. All the test subjects were instructed to take dinner before 21:00, and to set an interval of 3 h or more between daily meals (breakfast, lunch, and dinner). In addition, the subjects were allowed to consume snacks at up to 200 kcal per day, and alcohol consumption was limited to less than one large bottle of beer (500

mL), or its equivalent.

The daily diet, test food ingestion, rational symptoms, amount of exercise, and drug, functional food, and supplement intake of the test subjects were surveyed by asking them to describe each of these factors in a subject diary every day. The amount of exercise was checked by pedometer. Seven d before an examination (at 0, 4, 8, 12, and 16 w), a nutritionist checked the diets of all the test subjects and estimated the total energy, protein, fat, carbohydrate, and dietary fiber for each.

Statistical analysis

Values were expressed as mean $\pm$ SD. The degree of change in the fat area data from the CT scans was calculated for three study groups: total, male, and female. Repeated-measures analysis of variance (ANOVA) was used to compare differences among the groups. When significant differences were detected, Dunnett's test was used for multiple comparisons (*vs.* the placebo). In addition, a repeated-measures analysis of variance (ANOVA) was used to compare differences among periods (in each group). When significant differences were detected, Dunnett's test was used for multiple comparisons (*vs.* 0 w). A *p*-value ($p < 0.05$) was used as the criterion for statistically significant differences. All statistical analyses were performed using SPSS ver 16.0 (SPSS Japan, Tokyo).

Results

One subject dropped out of the study due to personal problems not related to the study. In addition, among the 89 remaining subjects, eight subjects were excluded from analysis for evaluation, due to BMI < 25 kg/ m^2 at 0 weeks (two subjects), irregular diet (five subjects), and taking supplements related to lipid metabolism (one subject). The final subject numbers in the three study groups were as follows: placebo group (n = 25; male = 12, female = 13), 200 mg PFE intake group (n = 28; male = 13, female = 15), and 300 mg PFE intake group (n = 28; male = 14, female = 14). The general characteristics of the study subjects are shown in Table 2. There were

no significant differences between either of the PFE intake groups and the placebo group.

Table2. Baseline Characteristics of Study Subjects (0 week)

Parameter		300 mg	200 mg	Placebo
Total		28	28	25
Male		14	13	12
Female		14	15	13
Age (years)	Total	43.4 ± 7.8	44.2 ± 7.5	44.0 ± 10.6
	Male	43.7 ± 8.9	43.2 ± 5.9	40.5 ± 9.8
	Female	43.0 ± 6.7	45.1 ± 8.8	47.3 ± 10.7
Height (cm)	Total	165.2 ± 7.7	164.3 ± 7.0	165.8 ± 9.0
	Male	170.3 ± 5.1	169.8 ± 5.2	173.1 ± 6.8
	Female	160.1 ± 6.5	159.5 ± 4.5	159.1 ± 4.2
Body weight (kg)	Total	75.3 ± 6.9	74.3 ± 7.6	75.7 ± 9.4
	Male	77.8 ± 5.3	78.7 ± 7.8	82.6 ± 7.6
	Female	72.9 ± 7.6	70.5 ± 5.2	69.3 ± 5.6
BMI	Total	27.6 ± 1.5	27.5 ± 1.5	27.4 ± 1.5
	Male	26.8 ± 1.2	27.3 ± 1.9	27.6 ± 1.7
	Female	28.4 ± 1.5	27.7 ± 1.3	27.3 ± 1.3
Visceral fat area (cm^2)	Total	116.4 ± 35.0	108.0 ± 49.1	95.3 ± 31.9
	Male	124.6 ± 40.7	127.4 ± 62.6	105.5 ± 39.8
	Female	108.3 ± 27.3	91.2 ± 25.1	86.0 ± 19.5
Subcutaneous fat area (cm^2)	Total	210.9 ± 60.1	219.5 ± 66.8	228.2 ± 45.0
	Male	165.4 ± 40.1	184.3 ± 37.4	218.4 ± 55.2
	Female	256.4 ± 37.8	250.1 ± 72.5	237.2 ± 32.9
Total fat area (cm^2)	Total	327.3 ± 59.8	327.5 ± 71.3	323.5 ± 58.4
	Male	290.0 ± 48.7	311.7 ± 75.1	323.9 ± 73.7
	Female	364.7 ± 45.2	341.3 ± 67.3	323.2 ± 43.0
Waist circumference (cm)	Total	95.9 ± 4.2	96.2 ± 6.3	96.0 ± 3.9
	Male	93.9 ± 4.1	95.0 ± 6.9	96.0 ± 4.1
	Female	98.0 ± 3.5	97.3 ± 5.6	95.9 ± 3.9
Hip circumference (cm)	Total	100.6 ± 4.5	100.1 ± 4.2	100.9 ± 4.4
	Male	99.0 ± 3.4	99.1 ± 3.7	100.8 ± 4.0
	Female	102.3 ± 4.9	100.9 ± 4.5	101.0 ± 4.9

Data are expressed as mean ± SD.

Table3. Dietary Composition and Exercise

Parameter	Group	n	0 w	4 w	8 w	12 w	16 w
Energy (kcal)	300 mg	28	1908 ± 271	1940 ± 252	1987 ± 287	1918 ± 248	1846 ± 270
	200 mg	28	1938 ± 341	2029 ± 318	1998 ± 284	1996 ± 276	1894 ± 313
	Placebo	25	1989 ± 380	2030 ± 287	2026 ± 378	2033 ± 347	1954 ± 344
Protein (g)	300 mg	28	68.1 ± 10.5	67.6 ± 9.0	68.7 ± 10.1	68.4 ± 9.4	65.1 ± 10.3
	200 mg	28	70.3 ± 13.7	71.4 ± 10.5	69.4 ± 9.7	71.2 ± 9.5	69.5 ± 13.6
	Placebo	25	71.6 ± 14.9	72.4 ± 11.1	72.3 ± 14.9	74.6 ± 14.3	72.6 ± 15.2
Fat (g)	300 mg	28	65.8 ± 14.1	61.4 ± 10.7	62.2 ± 10.5	60.5 ± 10.5	62.4 ± 12.5
	200 mg	28	68.6 ± 16.6	62.7 ± 14.6	63.3 ± 11.6	64.5 ± 12.1	63.0 ± 16.6
	Placebo	25	68.3 ± 15.9	61.9 ± 12.8	64.3 ± 15.7	66.2 ± 14.1	65.3 ± 14.9
Carbohydrate (g)	300 mg	28	247.3 ± 37.5	266.6 ± 42.4 **	274.9 ± 42.3 ***	261.5 ± 36.8	242.7 ± 44.6
	200 mg	28	245.1 ± 41.9	281.3 ± 42.8 ***	275.4 ± 39.7 ***	269.7 ± 42.5 ***	249.3 ± 40.9
	Placebo	25	258.0 ± 53.6	283.2 ± 40.9 ***	276.9 ± 44.8 *	270.7 ± 45.8	253.8 ± 45.8
Dietary fiber (g)	300 mg	28	11.6 ± 2.5	13.0 ± 1.8 **	14.4 ± 2.0 ***	13.8 ± 2.2 ***	10.9 ± 2.4
	200 mg	28	11.9 ± 2.6	14.3 ± 1.8 ***	15.3 ± 1.8 ***	14.6 ± 1.6 ***	11.6 ± 2.0
	Placebo	25	12.1 ± 3.3	14.1 ± 2.4 ***	14.8 ± 2.8 ***	14.2 ± 2.4 ***	11.5 ± 3.1
Pedometer count (steps)	300 mg	28	7818 ± 2764	8062 ± 2955	8139 ± 2962	8152 ± 2969	8338 ± 3052
	200 mg	28	9283 ± 3750	9230 ± 3567	9679 ± 4223	9600 ± 3546	9291 ± 3488
	Placebo	25	8976 ± 2320	8993 ± 2422	9297 ± 2694	9525 ± 2730	9268 ± 3191

Data are expressed as means ± SD.
*p<0.05, **p <0.01, ***p<0.001 *vs*. 0 w

Table4. Effects of PFE on Abdominal fat Area (Sex Stratifcated Analysis)

Parameter	Group	Sex	n	8 w		12 w	
⊿Visceral fat area (cm^2)	300 mg	Total	28	-8.9 ± 11.0	***#	-15.3 ± 11.1	***##
		Male	14	-12.3 ± 13.0	**	-16.7 ± 9.0	***#
		Female	14	-5.6 ± 7.5		-13.9 ± 13.1	***#
	200 mg	Total	28	-4.0 ± 6.1		-5.4 ± 12.1	**
		Male	13	-5.3 ± 4.4	*	-5.3 ± 6.4	*
		Female	15	-2.9 ± 7.1		-5.5 ± 15.7	
	Placebo	Total	25	-1.5 ± 10.4		-4.1 ± 11.2	
		Male	12	-2.6 ± 12.7		-9.1 ± 9.6	*
		Female	13	-0.5 ± 8.2		0.5 ± 10.9	
⊿Subcutaneous fat area (cm^2)	300 mg	Total	28	0.1 ± 24.4		-0.7 ± 32.2	
		Male	14	1.1 ± 20.5		-5.9 ± 32.4	
		Female	14	-0.8 ± 28.6		4.5 ± 32.2	
	200 mg	Total	28	0.9 ± 28.2		4.9 ± 33.5	
		Male	13	-2.7 ± 14.8		-2.3 ± 23.1	
		Female	15	3.9 ± 36.4		11.2 ± 40.2	
	Placebo	Total	25	-3.9 ± 27.7		1.5 ± 29.0	
		Male	12	-9.2 ± 19.4		-10.6 ± 21.4	
		Female	13	1.0 ± 33.7		12.6 ± 31.4	
⊿Total fat area (cm^2)	300 mg	Total	28	-8.8 ± 26.0		-16.0 ± 36.5	**
		Male	14	-11.2 ± 23.3		-22.6 ± 34.8	**
		Female	14	-6.4 ± 29.2		-9.4 ± 38.2	
	200 mg	Total	28	-3.2 ± 27.2		-0.5 ± 34.4	
		Male	13	-8.0 ± 15.7		-7.6 ± 25.7	
		Female	15	1.0 ± 34.3		5.7 ± 40.4	
	Placebo	Total	25	-5.4 ± 26.2		-2.6 ± 31.4	
		Male	12	-11.8 ± 19.6		-19.7 ± 23.8	**
		Female	13	0.4 ± 30.7		13.2 ± 29.8	

Data are expressed as means ± SD.
*p<0.05, **p <0.01, ***p<0.001 *vs*. 0 w
$^{\#}p$<0.05, $^{\#\#}p$<0.01 *vs*. placebo

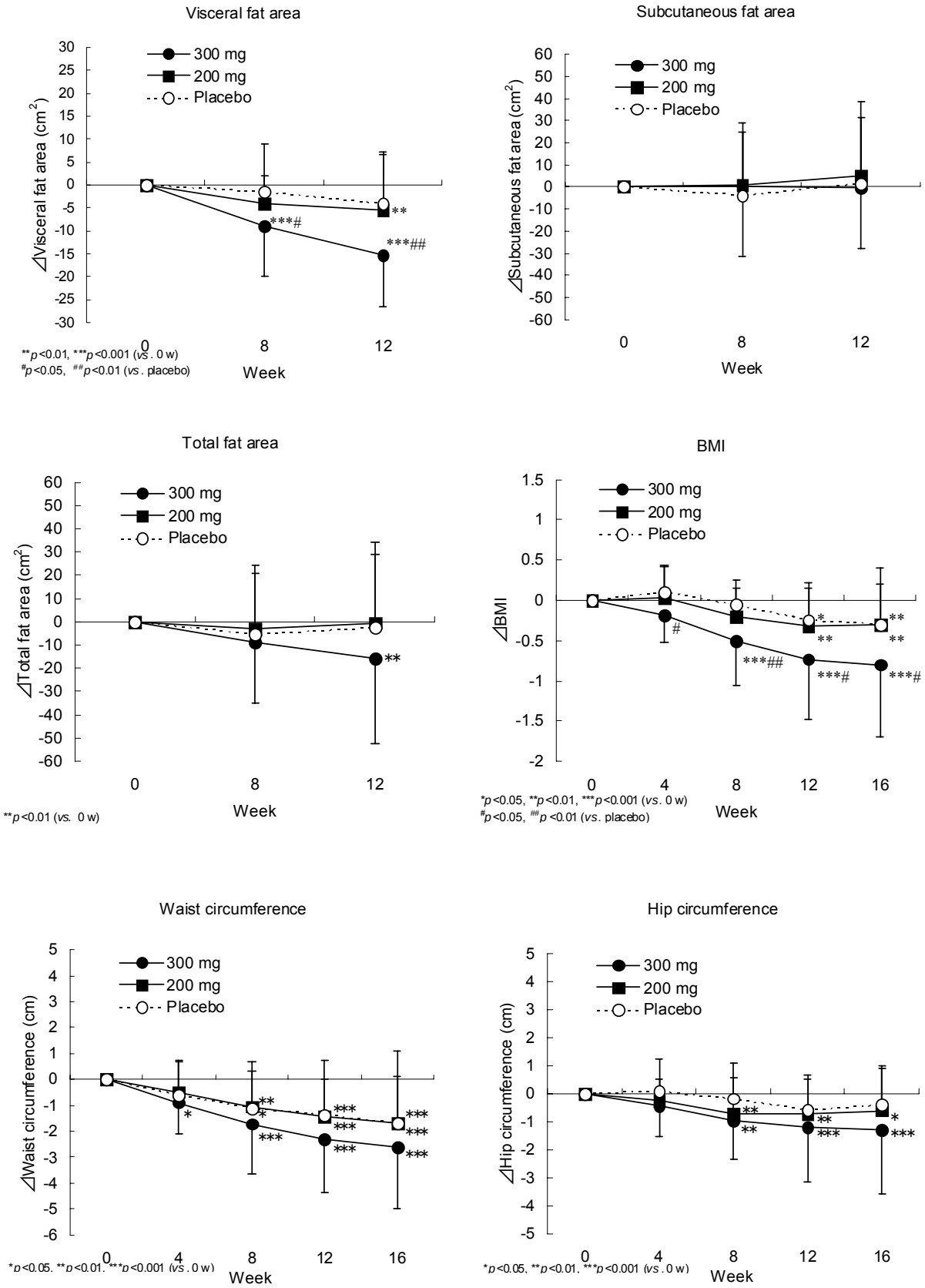

Figure1. Degrees of Change in Each of the Various Parameters

Table 3 shows the average values for dietary composition (total energy, protein, fat, carbohydrates, and dietary fiber intake) and pedometer count at 0, 4, 8, 12, and 16 weeks. There were no significant differences between either of the PFE intake groups and the placebo group, on any parameter. These data indicate that the study groups did not differ in daily energy, nutrient intake, or amount of exercise during the study.

First we investigated the effects of PFE intake on physical parameters including BMI, and hip and waist circumferences. In addition, the visceral, subcutaneous, and total fat areas were also detected by CT. Figure 1 shows the degrees of change, value at 0 w (baseline) = 0.0, in visceral fat area, subcutaneous fat area, total fat area (0, 8 w, and 12 w), BMI, waist circumference, and hip circumference (0, 4, 8, 12 w, and 16 w) for 300 mg PFE, 200 mg PFE, and placebo intake. As for BMI and visceral fat area at 300 mg of PFE intake, the degrees of change in BMI were -0.5, -0.7, and -0.8 at 8, 12 w, and 16 w respectively, while those for visceral fat area were -8.9 and -15.3 cm^2 at 8 and 12 w, a significant difference as compared to 0 w. In addition, there were significant differences between the 300 mg PFE intake group and the placebo intake group at 4 w and 16 w (BMI only), and at 8 w and 12 w (both BMI and visceral fat area). In contrast, no significant decrease in subcutaneous fat area relative to 0 w and to the placebo group was observed. These data indicate that 300 mg of PFE intake reduces BMI *via* a decrease in visceral but not subcutaneous fat area. As for waist and hip circumference for the PFE intake groups, although significant reductions were observed chronologically, these were not significant as compared to the placebo group.

Table 4 shows the results of a gender stratification analysis of visceral fat area, subcutaneous fat area, and total fat area. In both the male and female 300 mg PFE intake groups, significant decreases as compared to 0 w and the placebo group were observed in visceral fat area at 12 w. These data indicate that the visceral fat area decrease effect of PFE intake displays no sexual dimorphism.

Table5. Changes in Blood Biochemical Parameters

Parameter	Group	n	0 w	4 w	8 w	12 w	16 w
Triglyceride (mg/dL)	300 mg	28	121.6 ± 59.1	111.5 ± 38.6	98.6 ± 37.3 *	96.2 ± 33.0 *	117.2 ± 52.0
	200 mg	28	112.6 ± 44.1	115.4 ± 71.8	104.1 ± 45.6	118.1 ± 56.7	110.4 ± 42.4
	Placebo	25	111.9 ± 54.0	117.6 ± 55.8	100.4 ± 43.6	113.8 ± 59.2	112.0 ± 45.6
Total cholesterol (mg/dL)	300 mg	28	204.1 ± 30.8	208.4 ± 32.0	204.6 ± 35.9	199.8 ± 29.8	205.0 ± 35.3
	200 mg	28	208.4 ± 37.8	212.9 ± 37.8	202.5 ± 33.2	204.1 ± 37.9	205.7 ± 40.3
	Placebo	25	204.0 ± 38.1	200.0 ± 33.4	199.1 ± 34.8	197.5 ± 33.4	205.7 ± 35.9
LDL cholesterol (mg/dL)	300 mg	28	136.8 ± 28.4	140.3 ± 30.0	137.4 ± 33.6	133.2 ± 28.7	134.2 ± 31.2
	200 mg	28	134.8 ± 34.7	136.4 ± 33.2	131.8 ± 31.5	130.8 ± 34.1	129.4 ± 34.1
	Placebo	25	129.6 ± 31.0	125.1 ± 26.1	128.4 ± 29.2	124.4 ± 25.5	130.3 ± 31.5
HDL cholesterol (mg/dL)	300 mg	28	50.5 ± 8.8	50.0 ± 7.7	50.4 ± 10.0	50.1 ± 9.2	49.6 ± 8.4
	200 mg	28	55.3 ± 10.8	54.5 ± 11.7	52.9 ± 10.4	52.8 ± 10.4	54.1 ± 9.8
	Placebo	25	55.5 ± 13.0	52.7 ± 11.9	52.3 ± 11.0	52.8 ± 11.7	53.6 ± 13.6
Glucose (mg/dL)	300 mg	28	91.0 ± 7.3	92.8 ± 9.2	92.2 ± 6.6	91.2 ± 9.4	92.3 ± 5.9
	200 mg	28	93.7 ± 7.5	93.1 ± 8.7	94.4 ± 8.8	93.8 ± 9.0	94.1 ± 8.3
	Placebo	25	90.8 ± 7.4	89.9 ± 7.2	90.9 ± 5.5	89.3 ± 5.5	92.3 ± 5.5
HbA_{1c} (%)	300 mg	28	5.20 ± 0.23	5.02 ± 0.23 ***	4.99 ± 0.22 ***	4.99 ± 0.19 ***	5.01 ± 0.22 ***
	200 mg	28	5.20 ± 0.31	5.06 ± 0.28 ***	5.03 ± 0.28 ***	5.01 ± 0.28 ***	5.03 ± 0.30 ***
	Placebo	25	5.12 ± 0.46	4.96 ± 0.44 ***	4.94 ± 0.44 ***	4.93 ± 0.44 ***	4.97 ± 0.42 ***
Insulin (μU/mL)	300 mg	27	6.65 ± 2.51	7.66 ± 6.83	7.14 ± 2.95	6.63 ± 2.59	7.14 ± 2.26
	200 mg	27	5.65 ± 2.07	5.40 ± 1.77	5.58 ± 2.40	6.16 ± 2.47	6.24 ± 2.22
	Placebo	25	5.58 ± 1.82	5.00 ± 1.70	5.95 ± 2.03	5.63 ± 1.66	6.18 ± 2.49
AST (IU/L)	300 mg	28	19.6 ± 3.9	18.9 ± 4.5	19.2 ± 5.8	20.0 ± 9.6	20.1 ± 5.9
	200 mg	28	19.1 ± 5.3	20.7 ± 7.9	17.9 ± 4.5	19.1 ± 7.4	19.1 ± 3.9
	Placebo	25	18.7 ± 3.5	18.6 ± 4.8	18.1 ± 4.6	18.0 ± 5.0	19.3 ± 5.7
ALT (IU/L)	300 mg	28	26.4 ± 11.2	23.4 ± 10.4	23.8 ± 11.0	25.0 ± 18.9	23.8 ± 11.5
	200 mg	28	21.1 ± 10.7	20.6 ± 10.2	18.5 ± 8.6	21.1 ± 15.8	18.9 ± 6.5
	Placebo	25	23.6 ± 12.6	23.4 ± 14.4	21.5 ± 13.6	20.4 ± 10.1	23.4 ± 12.7
γGTP (IU/L)	300 mg	28	31.4 ± 12.5	26.6 ± 8.5 **	26.4 ± 10.5 **	26.6 ± 9.3 **	26.8 ± 7.6 **
	200 mg	28	28.3 ± 15.3	28.2 ± 15.0	26.7 ± 14.8	32.1 ± 32.4	28.4 ± 15.7
	Placebo	25	28.3 ± 16.6	27.8 ± 18.1	26.4 ± 16.8	26.2 ± 15.4	29.3 ± 16.9
Leptin (ng/mL)	300 mg	Male14	4.84 ± 1.31	3.64 ± 1.80 **	3.94 ± 1.99 *	3.51 ± 2.50 **	4.23 ± 1.83
		Female14	12.28 ± 2.81	12.19 ± 3.42	12.45 ± 3.81	10.46 ± 2.97	11.48 ± 3.81
	200 mg	Male13	5.38 ± 1.99	4.75 ± 1.66	4.75 ± 2.12	4.31 ± 1.83	4.98 ± 1.77
		Female15	12.03 ± 3.44	11.03 ± 3.28	12.08 ± 3.49	10.80 ± 3.80	13.14 ± 3.55
	Placebo	Male12	6.13 ± 2.72	5.00 ± 1.78 *	4.73 ± 1.99 **	4.29 ± 1.94 ***	5.07 ± 2.28
		Female13	11.38 ± 2.62	11.57 ± 2.71	11.30 ± 2.15	10.72 ± 3.32	11.21 ± 2.94
Adiponectin (μg/mL)	300 mg	28	5.35 ± 2.58	5.34 ± 2.88	5.60 ± 2.70	5.74 ± 3.26	
	200 mg	28	6.35 ± 2.62	6.41 ± 2.88	6.33 ± 2.95	6.28 ± 2.80	
	Placebo	25	7.35 ± 2.83	7.11 ± 2.99	7.99 ± 3.37	7.88 ± 3.51	

Data are expressed as means ± SD.
*p <0.05, **p <0.01, ***p <0.001 *vs*. 0 w
In the case of insulin, two people were eliminated from analysis because their values were too low to detect. Therefore, the numbers for the groups were 27 (300 mg PFE group), 27 (200 mg PFE group), and 25 (placebo group).

Next we investigated other effects of PFE intake. Table 5 shows the results of a biochemical blood test. They indicate that there were no significant differences between either of the PFE intake groups and the placebo group on any parameter. Triglyceride and γGTP levels were significantly

lower than baseline (0 w) only in the 300 mg PFE intake group. It appears that PFE intake also has weak effects on the normalization of triglycerides and as a hepatoprotectant. In the case of HbA_{1c}, significant reductions relative to 0 w were observed in both PFE intake groups and in the placebo group. Considering the chronological reduction in the placebo group, it is difficult to draw conclusions as to the relationship between PFE intake and sugar metabolism. In addition, we checked hormone levels in the blood, including leptin and adiponectin, because PFE intake reduces body fat mass. Leptin levels chronologically decreased in the male 300 mg PFE intake group, but a similar reduction was observed in the placebo group. There was no significant increase in adiponectin levels in either PFE intake group.

Finally, throughout the entire period of the study, no adverse events directly related to PFE intake were reported.

Discussion

Flavonids are present in vegetables, fruits, tea, and wine, and have a diversity of functions, as anti-oxidants, for example. Isoflavone is categorized as a flavonid. Daidzein, genistein, glyctin are well-known as soy isoflavones, and many clinical and animal studies have reported that intake of soy isoflavones improves the blood lipid profile and glucose metabolism, and thus is beneficial in several chronic disorders associated with obesity and diabetes [13]. Recently, animal studies have evaluated the effects of soy isoflavones on body weight and lipid profile. It has been reported that daidzein and genistein suppressed weight gain and altered hepatic gene expression profiles, as for lipolysis and lipogenesis, as well as adipocyte metabolism [13-15]. We have reported that PFEs possess anti-obesity and anti-fatty liver effects through suppressing lipogenesis in the liver and promoting lipolysis in white adipose tissue and thermogenesis in brown adipose tissue, similarly to soy isoflavones such as daidzein [13, 16]. In addition, it has been reported that *Pueraria* flowers are a rich source of isoflavones. *Pueraria thomsonii* contains seven isoflavones, tectoridin, tectorigenin 7-*O*-xylosylglucoside, 6-hydoroxygenistein-6,7-diglucoside, glycitin (glucosides), and tectorigenin, glycitein, and genistein (aglycones) [7]. As a quantitative analysis using HPLC, the PFEs used in this study contained all these isoflavones, and the total amount of isoflaveone in the PFE was

approximately 18% (see “Materials and Methods”). Moreover, the isoflavone-rich fraction (total isoflavone amount, 63%) of *Pueraria thomsonii*, as well as the PFE, had a body weight reduction effect in the mice (unpublished data). Hence, we speculate that the body weight reduction effect of PFE observed in this study is due primarily to isoflavones, and that the mechanism of fat reduction is an alteration of the hepatic or adipocytic gene profile.

In this study, we found that PFE intake over 12 weeks reduced BMI *via* a decrease in visceral fat area, with no sexual dimorphism. The body weight and fat reduction effects were observed for a daily oral intake of 300 mg of PFE, converted to an intake of 54 mg of isoflavones per day. Considering that some of the Zutphen Elderly Studies have indicated that a daily flavonoid intake of more than 30 mg reduces the risk of death from coronary heart disease for elderly men [17, 18], a dose of 300 mg of PFE might be correct. In contrast, although a dose of 200 mg of PFE also induced sequential reduction of BMI and visceral fat area as compared to 0 w, there were no significant differences as compared to the placebo group. These data suggest that 300 mg of PFE is the optimal effective dose for reduction of visceral fat.

It is also well-established that absorption of isoflavones from the diet requires deglycosylation (the conversion of glycosides to aglycones) [19]. Tsuchihashi *et al*., as well as our group have reported that the major glycosides of *Pueraria thomsonii* are metabolized to tectorigenin by anaerobic cultivation by human fecal or human intestinal bacterial strains [20, 21]. It is likely that aglycone (tectorigenin), derived from *Pueraria thomsonii*, is a key factor in the reduction of body weight and fat observed in this study. In future work, we intend to investigate the active components of PFE in detail.

A metaanalysis has suggested that soy isoflavones lower total and LDL cholesterol in humans [22], but in the present study no reduction in total or LDL cholesterol with PFE intake was observed. It has been reported that hormonal estrogen improves cholesterol homeostasis [23]. Tectorigenin, probably a main metabolite from PFE, had low binding activity to estrogen receptor α and β as compared to soy isoflavones such as genistein. Possibly due to this weak estrogenic effect of tectroigenin as compared to soy isoflavones, no cholesterol homeostasis was observed.

In this study, we detected a reduction in γGTP as compared to 0 w in the 300 mg PFE intake

group. Tectoridin and tectorigenin reduced the ALT and AST values increased by *tert*-butyl hyperoxide (*t*-BHP) [24]. Decreases in the γGTP concentration were observed in our previous studies [12]. Hence, PFE may also help in liver protection in humans. In addition, triglyceride reduction was observed as compared to 0 w in the 300 mg PFE intake group. In our previous animal study, PFE intake reduced ACC gene expression in the liver and increased UCP1 gene expression in the brown adipose tissue [16]. Some clinical studies have found that suppression of lipogenesis and mutation of UCP1 influence serum triglyceride levels [25, 26]. The reduction of trigriceride observed in this study might have been due to changes in these gene profiles.

Previously we reported the effect on body weight reduction of PFE intake in males [12]. In the present study, we investigated the effect of PFE intake on obesity in both males and females. We found that 300 mg of PFE intake over 12 weeks reduced BMI *via* a decrease in visceral fat area, with no sexual dimorphism. Moreover, in this study, no serious adverse events directly related to PFE intake were reported. We propose that PFE extracted from flowers of *Pueraria thomsonii* might serve as a functional food promoting body fat reduction.

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Chapter 3

Studies on Anti-obesity Mechanisms of *Pueraria* Flower Extract

Section 1

Evaluation of the Estrogenic Activity of *Pueraria* Flower Extract and Its Major Isoflavones Using Estrogen Receptor Binding and Uterotrophic Bioassays

Introduction

Pueraria flower extract (PFE) is a hot water extract of the Kudzu flower (*Pueraria thomsonii*). Kudzu is a leguminous plant that grows in Japan, China, and other countries. The Kudzu flower is frequently used in traditional Chinese medicine for counteracting symptoms associated with alcohol use, liver injury, and menopause. In China, tea made from dried Kudzu flowers is widely consumed [1], and PFE is consumed as a nutritional supplement for the treatment of hangovers and obesity in Japan. We previously reported that PFE exerts anti-obesity effects in humans and mice fed high-fat diets [2-4]. In addition, we also reported that the isoflavone-rich fraction of dietary PFE exhibits anti-obesity effect in mice fed high-fat diets [5].

The following isoflavones are found in the Kudzu flower but not in soy: 6-hydroxygenistein 6,7-di-*O*glucoside (6HGDG), tectorigenin 7-*O*-xylosylglucoside (TGXG), tectoridin, genistin, glycitin, tectorigenin, genistein, and glycitein [5]. Among these isoflavones, 6HGDG, TGXG, tectoridin and tectorigenin, which comprise nearly 90% of the isoflavones in the Kudzu flower, are present in PFE. Previous studies have shown that the hydrolysis of the sugar moiety is required for isoflavone bioavailability and that isoflavone glycosides are not absorbed intact across the enterocyte in healthy adults [6]. When cultured with enteric bacteria, tectoridin and TGXG are metabolized into tectorigenin and 6HGDG is metabolized into the aglycone isoflavone 6-hydroxygenistein [7-8]. Consequently, the major isoflavones in PFE are likely to be absorbed into the bloodstream in the forms of tectorigenin and 6-hydroxygenistein.

Isoflavones are polyphenolic compounds that are capable of exerting estrogen-like effects. For this reason, isoflavones are classified as phytoestrogens. Due to their conformational similarity to β-estradiol, isoflavones exbihit similar effects on estrogen receptors (ERs). Phytoestrogens have recently received great attention because of their beneficial effects, including the prevention of atherosclerosis [9], and bone density loss [10]. In particular, recent studies revealed that ERs affect adipose tissue weight. Namely, ERα knockout mice show increased fat pad weight and adipocyte size and insulin resistance [11]. In addition, the ER agonist is reported to exert an anti-obesity effect without eliciting any untoward effects on the mammary gland or uterus in mice [12].

A previous study demonstrated that the high binding affinities of select isoflavones is associated with the structural properties of these molecules. For example, the binding affinities of select molecules for ER-α and -β were observed in the following order: genistein > daidzein > biochanin A > glycitein > formononetin A (ER-α); and genistein > glycitein > daidzein > formononetine > biochanin A (ER-β) [13]. Based on these results, soy isoflavones, especially genistein are thought to exhibit strong estrogenic activity. For these reasons, to obtain the relative estrogenic activities against soy isoflavone is thought to be valuable.

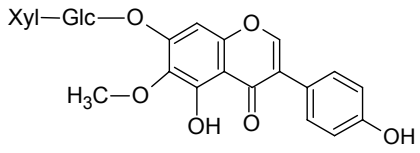
In this study, we conducted ER-binding assays to investigate the relative estrogenic activities of 6-hydroxygenistein and tectorigenin, the aglycone forms of PFE's major isoflavones as well as uterotrophic bioassays to investigate the estrogenic effects of PFE *in vivo*.

Materials and Methods

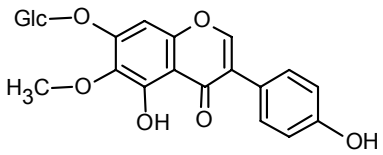
Experimental materials

PFE was purchased from Ohta's Isan Co. Ltd. (Ibaraki, Japan). PFE contains TGXG (8.5%), tectoridin (4.6%), 6HGDG (3.4%), tectorigenin (0.9%), genistin (0.3%), glycitin (0.2%), glycitein (0.1%), and genistein (0.1%). Quantitative analysis methods of isoflavones in PFE have been reported by Kamiya T *et al.* (2012c). Soy isoflavone product (SOY) was obtained from Nippon Bulk Yakuhin Co., Ltd. (Osaka, Japan). SOY containes 42.7% isoflavones (daidzein, daidzin, and a

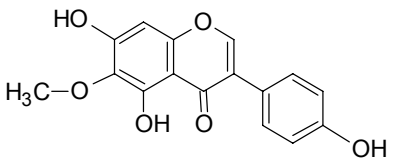
daidzin derivative, 23.3%; genistein, genistin, and a genistin derivative, 5.6%; glycitein, glycitin, and a glycitin derivative, 13.2%). The dietary soy isoflavone-aglycone (aglycone equivalent) testing method (see Food Safety Notification No. 0823001, August 23, 2006 for guidelines regarding the handling of select health foods, including soy isoflavones) was used to perform a quantitative analysis of isoflavones present in SOY. Tectorigenin, 6-hydroxygenistein, and genistein were purchased from Tokiwa Phytochemical Co., Ltd. (Chiba, Japan). Equol was purchased from ChromoDex, Inc (California, USA), and ethinylestradiol was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Figure 1 shows the chemical structures of Kudzu isoflavones.



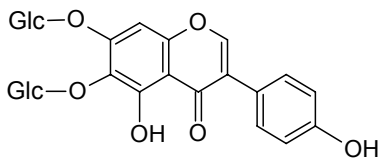
Tectorigenin 7-O-xylosylglucoside



Tectoridin



Tectorigenin



6-hydroxygenistein 6,7-di-O-glucoside

Figure1. Chemical structures of tectorigenin 7-O-xylosylglucoside (TGXG), tectoridin, tectorigenin, and 6-hydroxygenistein 6,7-di-O-glucoside (6HGDG)

ER-binding assays

The binding affinity of phytoestrogens to ER-α and -β was measured using the EnBio estrogen receptor cofactor assay system according to the manufacturer's protocol (EnBio Tec Laboratories, Japan). Briefly, we added biotinylated coactivator peptide to an avidin-coated plate and incubated the plate for 1 h at room temperature. We mixed estrogenic compounds, such as β-estradiol (provided by the manufacturer), tectorigenin, 6-hydroxygenistein, genistein, and equol

with recombinant human ER-α and -β. Then, the mixtures of ER-α and -β were added to the biotinylated coactivator-coated plates, which were subsequently incubated for 1 h at room temperature. After washing the plate three times with the wash buffer provided by the manufacturer, anti-ER-α and –β antibodies linked to HRP were applied to each well. After incubating the mixture for 30 min at room temperature, the plate was washed three times and a 3, 3', 5, 5'-tetramethylbenzidine substrate solution was added. The absorbance of each well was measured at 450 nm using a Powerscan HT (DS Pharma Biomedical Co., Ltd., Japan).

Uterotrophic bioassays

Uterotrophic bioassays were performed in accordance with the OECD Guidelines for the Testing of Chemicals (Uterotrophic bioassays in rodents. a short-term screening test for oestrogenic properties; No.440, adopted on October 16, 2007). All procedures involving animals were performed in accordance with the guidelines for the animal experiments at CERI Hita according to Laboratory Animal Science (1987) by the American Association for Laboratory Animal Science and were approved by the Ethical Committee of the Chemicals Evaluation and Research Institute (Oita, Japan). Briefly, female Sprague-Dawley rats ovariectomized at the age of 6 weeks were purchased from Japan SLC Inc. (Sizuoka, Japan) at the age of 7 weeks. After being acclimated for 1 week, each Sprague-Dawley rat was weighed and assigned to one of eight groups in order for each group to have a uniform mean body weight. Rats were given daily po injections of either purified water (vehicle control), PFE (250, 500, and 1000 mg/kg), SOY (250, 500, and 1000 mg/kg), or s.c. injections of ethinylestradiol (EE, 0.6 μg/kg; positive control) for 7 days. At the end of treatment, the rats were sacrificed, and each uterus and vagina was removed. The wet and blotted uterus weights were measured, and macroscopic examinations of the uterus and vagina were performed.

Data were expressed as the mean ± SD. The body and uterus weights were initially analyzed using Bartlett's test of homogeneity of variance. When the results were determined to not be significant, one-way ANOVA was performed. After statistically significant differences were

determined, a post-hoc pairwise comparison was performed using Dunnett's test, in which vehicle control groups were compared to either PFE or SOY groups. When the results of Bartlett's test were significant, treated and control groups was compared using the Kruskal-Wallis test and non-parametric type Dunnett's test. For comparisons between vehicle and positive control groups, the body and uterus weights were initially analyzed using the F-test. When the results of the F-test were not significant, a student's *t*-test was performed. However, when the results of the F-test were determined to be significant, the Aspin-Welch's *t*-test was subsequently performed.

Results

ER-binding assay

The ER-binding curves for the 5 compounds are shown in Figure 2. Table 1 lists the EC_{50} values of each chemical and their relative binding affinities. The binding affinities for ER-α and -β were observed in the following order; β-estradiol > genistein > equol > tectorigenin > 6-hydroxygenistein. The relative binding affinities of tectorigenin for ER-α and -β were 0.04 and 0.02 that of genistein, respectively, and the relative binding affinity of 6-hydroxygenistein to ER-α was 0.01 that of genistein. However, an EC_{50} value for 6-hydroxygenistein could not be determined with ER-β.

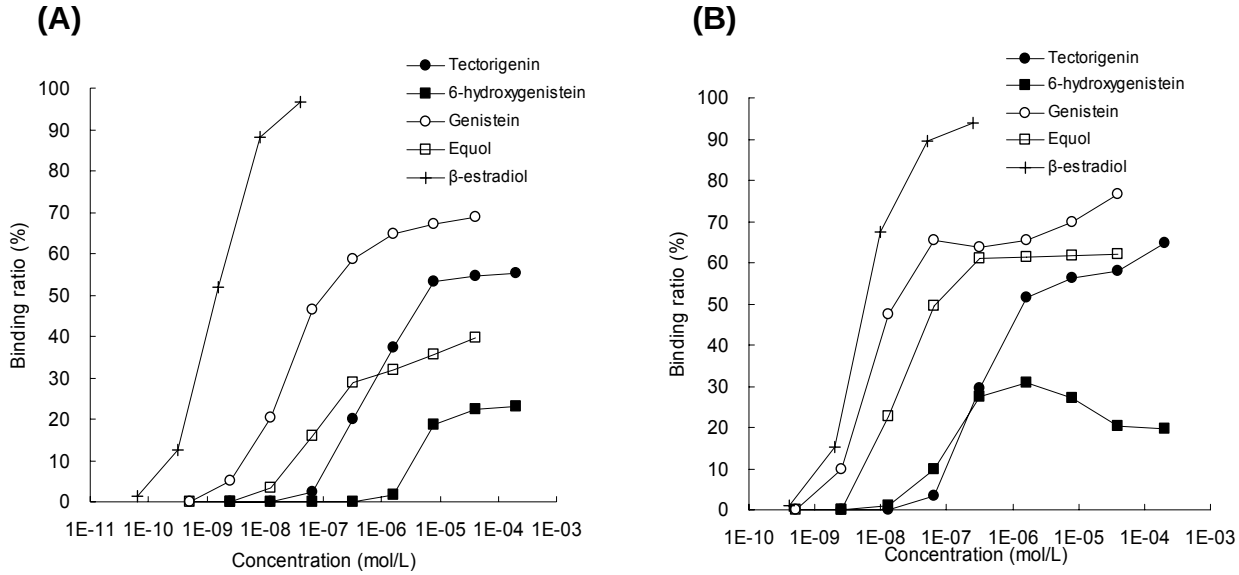


Figure2. The ER-binding curves for the test substrates

Table1. Binding affinities of test substrates for human estrogen receptors-α and -β

	EC_{50}		Relative binding affinity			
	ER-α	ER-β	vs β-Estradiol		vs Genistein	
	(mol/L)	(mol/L)	ER -α	ER- β	ER -α	ER -β
β-Estradiol	1.5×10^{-9}	5.5×10^{-9}	100.0	100.0	186.7	136.4
Genistein	2.8×10^{-8}	7.5×10^{-9}	5.4	73.3	100.0	100.0
Equol	8.9×10^{-8}	19×10^{-9}	1.7	28.9	31.5	2.5
Tectorigenin	65×10^{-8}	340×10^{-9}	0.2	1.6	4.3	2.2
6-hydroxygenistein	200×10^{-8}	NC	0.1	NC	1.4	NC

Uterotrophic bioassays

There were no significant differences in the final body weight among the groups (data not shown).

In the both SOY groups (500 mg/kg and 1000 mg/kg), the wet and blotted uterus weights were significantly higher than those of the control group (Table 2). Additionally, macroscopic examination revealed that the thickening of the uterine wall was observed in SOY groups (500 mg/kg and 1000 mg/kg: Table 3). On the other hand, no abnormalities were observed in the uterine weights and macroscopic examination of PFE groups (250 mg/kg, 500 mg/kg, and 1000 mg/kg).

Table2. Results of absolute uterus weight

		Wet uterus (mg)		Blotted uterus (mg)	
Control		82.5 ± 9.6		81.2 ± 9.3	
PFE	250 mg/kg	84.4 ± 24.5		82.8 ± 23.2	
	500 mg/kg	81.4 ± 7.4		80.4 ± 7.5	
	1000 mg/kg	90.5 ± 16.5		89.3 ± 16.7	
SOY	250 mg/kg	92.8 ± 11.0		91.6 ± 11.0	
	500 mg/kg	116.3 ± 18.9	**	114.7 ± 18.5	**
	1000 mg/kg	160.2 ± 20.8	**	156.0 ± 21.7	**
EE	0.6 ug/kg (s.c.)	321.8 ± 31.2	**	305.0 ± 22.7	**

Data are expressed as the mean ± SD. The values marked with asterisks differ significantly from the control group (**, $p<0.01$)

Table3. Summary of macroscopic examination

		PFE			SOY			EE
	Control	250 mg/kg	500 mg/kg	1000 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg	0.6 ug/kg (s.c.)
No abnormalities	6	6	6	6	6	5	0	0
Uterus								
Thickening of wall	0	0	0	0	0	1	6	6
Vagina								
Retention of mucus in vaginal cavity	0	0	0	0	0	0	0	1

Discussion

The results of the uterotrophic bioassays demonstrate that PFE cannot exert estrogenic activity, even at a dose of 1000 mg/kg/day, which is the limit dose designated in the current draft protocol by

the OECD. On the other hand, SOY appears to exert estrogenic activity.

In this study, the non-observed effect level (NOEL) of SOY was considered to be 250 mg/kg (equivalent to 107 mg/kg soy isoflavones), and the lowest observed effect level (LOEL) was also considered to be 500 mg/kg in uterotrophic bioassays (equivalent to 214 mg/kg soy isoflavones). The soy isoflavone genistein was reported to induce an increase in uterine wet weight at a dose of 100 mg/kg [14]. The test substrate used in the present study was a soy isoflavone mixture, which contained genistein, daidzein, and glycitein. The binding affinities of daidzein to ER-α and -β was 0.27 and 0.03 that of genistein, respectively. In addition, the binding affinities of glycitein to ER-α and -β were 0.15 and 0.03 that of genistein, respectively [13]. Notably, genistein exhibited the highest binding affinities for ERs compared to those of other soy isoflavones. Consequently, genistein can potentially induce increases in uterine wet weight at comparatively low dosages.

The respective binding affinities of the aglycone forms of the major isoflavones in PFE, tectorigenin and 6-hydroxygenistein, for ERs were the following: ER-α, 0.04 and 0.01 that of genistein; and ER-β, 0.02 and 0.00 that of genistein, respectively. The binding affinities of daidzein, glycitein, biochanin A, and formononetin for ER-α were reported to be 0.27, 0.15, 0.16, 0.03 those of genistein, respectively, and the binding affinities of daidzein, glycitein, biochanin A, formononetin for ER-β were 0.03, 0.03, 0.01, 0.02 those of genistein, respectively [13]. The relative binding affinities of genistein to the ER-α and -β in the present study were similar to the values previously reported. Briefly, tectorigenin and 6-hydroxygenistein have lower ER binding affinities compared to those of daidzein and biochanin A. It is reported that daidzein and biochanin A did not induce an increase in uterine wet weight at doses within the range of 0.00001 - 1000 mg/kg in uterotropic bioassays [14]. In the present study, PFE did not increase the uterine weight at a dose of 1000 mg/kg, because the major isoflavones in PFE only exhibit slight estrogenic activity.

Moreover, the present study suggests that PFE does not contain compounds that exhibit strong estrogenic activity. The legume plant *Pueraria mirifica* has been reported to contain miroestrol and deoxymiroestrol [15], and the extent of the estrogenic activity of these compounds are

approximately 4 and 20 times higher than that of genistein [16]. In particular, miroestrol promotes uterine and vaginal growth in the immature female mice [17], and *Pueraria mirifica* influences menstrual cycles and suppresses ovulation at doses of 10 mg/kg and 100 mg/day in female monkeys [18]. Therefore, if PFE contains compounds that exhibit strong estrogenic effects, such as miroestrol and deoxymiroestrol, PFE would have to be administered at considerably low dosages to cause an increase in uterine weight. In fact, we performed a comprehensive mass analysis of the components comprising PFE and miroestrol and deoxymiroestrol were not detected (unpublished data).

In conclusion, tectorigenin and 6-hydroxygenistein, the major isoflavones of PFE exhibit weak estrogenic effects. These results suggest that the mechanism of anti-obesity effect of PFE is not mediated by ERs. Further studies are needed to elucidate the PFE's action against adipose tissue.

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Section 2

Studies on the Gene Expression Related to Lipid Metabolism in High-fat Diet-fed Mice

Introduction

Puerariae flower extract (PFE) is a crued extract from the flowers of Kudzu (*Puerariae thomsonii*). It contains approximately 20 percent of isoflavones as the major ingredient. In Japan, PFE is utilized as nutritional supplement for treatment of hangovers and obesity.

Kudzu, a leguminous plant distributed in Japan, China, and other areas, has long been used in folk medicine. In particular, *Puerariae* flowers are used in Japanese and Chinese folk medicine for treating hangovers [1, 2, 3]. Niiho *et al.* confirmed that the *Puerariae lobata* flower exerts hepatoprotective effects in individuals with liver injury induced by carbon tetrachloride or a high-fat diet in animal studies [1, 3]. Recently, research of the effects of Kudzu on lipid metabolism and obesity was reported. Wang *et al.* confirmed that flavones derived from *Radix Puerariae* exert inhibitory effects on body weight, abdominal fat content, and lipid levels in the liver [4]. In addition, we preliminarily investigated the effect of PFE on body weight in humans and found that PFE intake might reduce body weight and abdominal fat content in mildly obese subjects [5]. However, the anti-obesity mechanism of PFE is not known.

Obesity is a well-established risk factor for the development of hypertension, diabetes, dyslipidemia, and cancers, and it causes premature death [6]. An increase in visceral fat is responsible for many of the metabolic abnormalities, such as impaired glucose tolerance, insulin resistance, and increased very low-density lipoprotein triglyceride (VLDL-TG) levels, associated with abdominal obesity [7, 8, 9]. In addition, it is reported that approximately 50% of cases of obesity involving visceral fat accumulation are complicated by fatty liver [10]. Fatty liver is a reversible condition in which triglycerides accumulate in large vacuoles in hepatocytes. Severe fatty liver is occasionally accompanied by inflammation, a situation that is referred to as steatohepatitis.

When inflammation and steatohepatitis occur in people who do not drink alcohol, the condition is called non-alcoholic steatohepatitis (NASH), and it is known to correlate strongly with obesity [11]. Sources of fatty acids stored in the liver are as follows: 59% of fatty acids are stored in white adipose tissue (WAT); 26% is produced by lipogenesis in hepatocytes; and 15% is obtained from the diet [12]. Accordingly, it is considered that both reducing WAT content and suppressing lipogenesis in the liver are very important for controlling fatty liver.

WAT is a specialized connective tissue that functions as the major storage site for fat in the form of triglycerides. For use as energy, triglycerides are metabolized into fatty acids and transported by the bloodstream to tissues such as the liver and brown adipose tissue (BAT). Therefore, to prevent obesity, it is important to stimulate lipolysis in WAT and increase energy utilization in the liver and BAT.

Thus, in this study, we conducted an animal study to investigate the effect of PFE on visceral fat levels and hepatic lipid accumulation in mice with diet-induced obesity. In addition, we focused on the expression profiles of genes related to beta-oxidation and lipogenesis in the liver, lipolysis in WAT, and thermogenesis in BAT to investigate the anti-obesity mechanism of PFE.

Materials and Methods

Experimental materials

PFE was purchased from Ohta's Isan Co. Ltd. (Ushiku city, Japan). PFE contains 7 isoflavones (4 isoflavone glucosides: tectoridin (4.70%), tectorigenin 7-*O*-xylosylglucoside (8.37%), 6-hydroxygenistein-6,7-diglucoside (3.38%), and glycitin (0.17%); and 3 aglycones: tectorigenin (0.83%), glycitein (0.10%), and genistein (0.06%)). All isoflavone standard preparations were purchased from Nagara Science Co., Ltd. (Gifu, Japan) and Tokiwa Phytochemical Co., Ltd. (Chiba, Japan).

Experimental animals

All procedures using animals were performed in accordance with the Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animal Science and were approved by the Ethical Committee of TOYO SHINYAKU Co., Ltd. Male C57BL/6J mice were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan) at the age of 6 weeks.

Test environment

During the acclimation period, mice were housed in polycarbonate animal cages (260 × 420 × 180 mm; CLEA Japan, Inc., Tokyo, Japan) in groups of 4 and administered the MF diet (Oriental Yeast Co. Ltd., Tokyo, Japan). During the test period, mice were housed in individual stainless-steel wire mesh cages (750 × 210 × 150 mm; Tokiwa, Tokyo, Japan) under a 12 h light:dark cycle.

Experimental design

After acclimation for 1 week, C57BL/6J mice were weighed and assigned to one of 2 groups so that the mean body weight of each group was uniform. The control group was given a high-fat diet (HFD), and the treatment group was given the HFD containing 5% PFE (HFD+PFE) (Table 1). The animals were restricted-fed and given water *ad libitum* for 14 days.

Table1. Composition of the experimental diets

Ingredient	HFD	HFD+PFE
Casein	20.0	20.0
Alpha-potato starch	28.2	23.2
Sucrose	13.0	13.0
Corn oil	20.0	20.0
Lard	10.0	10.0
Cellulose	4.0	4.0
Mineral mixture	3.5	3.5
Vitamin mixture	1.0	1.0
dl-Methionine	0.3	0.3
PFE	0.0	5.0
Total	100.0	100.0

Mineral mixture, AIN-76; Vitamin mixture, AIN-76

(g/100 g diet)

Body weight and food intake

During the experiment, the animals were weighed every 4 days. Food intake was determined daily by determining the amount of feed remaining from the previous day, and the mean daily food intake for each animal was calculated.

Measurement of fecal lipids

All feces were collected daily on days 11–14 after the start of the experiment. The collected feces were dried for at least 3 days at 100°C and weighed. After dry feces were crushed and homogenized, fecal lipids were extracted using the Folch extraction protocol [13]. The total lipid content was determined by measuring the total dry extract weight.

Measurement of tissue weight

Fourteen days after the start of the experiment, mice were sacrificed, and then the liver and interscapular brown, mesenteric, epididymal, and retroperitoneal adipose tissues were removed. All samples excluding BAT samples were weighed. Each sample was cut into small pieces, dipped in RNA-later (Ambion, Tokyo, Japan), and stored at -80°C until RNA extraction. Another piece of each liver sample was stored at -80°C for subsequent analysis.

Hepatic histological analysis

Oil Red-O staining was performed in frozen liver sections to detect the presence of fat. The degree of fatty liver was assessed by expert pathologists. We entrusted all analyses to Narabyouri Research Co., Ltd. (Nara, Japan).

Real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR)

The total RNAs from the liver, epididymal adipose tissue, and BAT were isolated using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's directions. Total RNA (1.0 μg) was reverse-transcribed into cDNA in a reaction mixture using the QuantiTect Reverse Transcription kit (QIAGEN) according to the manufacturer's directions. The gene expression levels in the liver, epididymal adipose tissue, and BAT were determined using a real-time PCR system (Mini-opticon™ System; Bio-Rad Laboratories, Inc., Tokyo, Japan), the QuantiTect SYBR Green PCR kit (QIAGEN), and specific sets of primers (Table 2). The relative gene expression level was calculated with real-time PCR data relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table2. Polymerase chain reaction primer sequences

Gene name	Function	Genebank ID	Direction	Sequences
Fatty acid synthase (FAS)	lipogenesis	NM_007988.3	Forward	TCCTGGGAGGAATGTAAACAGC
			Reverse	CACAAATTCATTCACTGCAGCC
Acetyl-CoA carboxylase (ACC)	lipogenesis	NM_133904.2	Forward	TGGATCCGCTTACAGAGACTTT
			Reverse	GCCGGAGCATCTCATTCG
Carnitine palmitoyltransferase1 (CPT1)	beta-oxidation	NM_013495.2	Forward	CTTCCAAGGCAGAAGAGTGG
			Reverse	GAACCTTGGCTGCGGTAAGAC
Medium-chain acyl dehydrogenase (MCAD)	beta-oxidation	NM_007382.4	Forward	TCGAAAGCGGCTCACAAGCAG
			Reverse	CACCGCAGCTTTCCGGAATGT
Acyl-CoA oxidase (ACO)	beta-oxidation	AF006688.1	Forward	TCTTCTTGAGACAGGGCCCAG
			Reverse	GTTCCGACTAGCCAGGCATG
Hormone-sensitive-lipase (HSL)	lipolysis	BC021642.1	Forward	CCTACTGCTGGGCTGTCAA
			Reverse	CCATCTGGCACCCTCACT
Uncoupling protein1 (UCP1)	thermogenesis	NM_009463.2	Forward	CTGGGCTTAACGGGTCCTC
			Reverse	CTGGGCTAGGTAGTGCCAGTG
PPARg coactivator alpha (PGC1a)	mitochondriogenesis	BC066868.1	Forward	TCGATGTGTCGCCTTCTTGC
			Reverse	ACGAGAGCGCATCCTTTGG
GAPDH	housekeeping	XM_001478412.1	Forward	ATGACATCAAGAAGGTGGTG
			Reverse	CATACCAGGAAATGAGCTTG

Statistical analysis

Data were expressed as the mean ± SEM. For comparisons between groups, analyses were performed using unpaired *t*-tests on all test items. All statistical analyses were performed using Statview ver. 5.0 (SAS Institute Japan Ltd., Tokyo, Japan), and significance was set at $p < 0.05$.

Results

Body weight and food intake

Table 3 shows the body weight and amount of food intake 14 days after the start of the experiment. There was no significant difference between the 2 groups regarding food intake. Conversely, both the final body weight and body weight gain in the HFD+PFE group were significantly lower than those in the HFD group.

Table3. Effects of PFE on food intake, body weight, liver weight, adipose tissue weight, and fecal total lipids

	HFD	HFD+PFE	
Food intake, g/day	2.83 ± 0.04	2.79 ± 0.11	
Final body weight, g	26.4 ± 0.5	24.9 ± 0.3	*
Body weight gain, g	2.8 ± 0.3	1.4 ± 0.3	**
Liver weight, g/100 g body weight	4.54 ± 0.08	4.73 ± 0.12	
White adipose tissue weight			
Epididymal, g/100 g body weight	3.22 ± 0.18	2.49 ± 0.14	**
Mesentric, g/100 g body weight	1.31 ± 0.07	1.11 ± 0.10	
Retroperitoneal, g/100 g body weight	0.36 ± 0.03	0.26 ± 0.02	*
Fecal total lipids, g/day	0.015 ± 0.001	0.017 ± 0.001	

a) The data represent the mean ± SEM values (n = 8).

b) * and ** indicate significantly different at $p < 0.05$, $p < 0.01$, respectively.

Liver and adipose tissue weight

Table 3 shows the liver weight and epididymal, mesenteric, and retroperitoneal adipose tissue weights on day 14 after the start of the experiment. There were no significant differences between the 2 groups regarding liver weight and mesenteric adipose tissue weight. However, epididymal and retroperitoneal adipose tissue weights in the HFD+PFE group were significantly lower than those in the HFD group. These data indicate that PFE reduces body weight by decreasing fat weight in high-fat diet-induced obese mice.

Fecal lipid levels

There was no significant difference between the 2 groups regarding fecal lipid levels (Table 3). In addition, food intake was not significantly different between the 2 groups (Table 3), and thus, it was believed that energy intake did not differ substantially between the 2 groups.

Hepatic histological analysis

Table 4 and Figure 1 show the hepatic histological analysis data on day 14 after the start of the

experiment. In the HFD+PFE group, the development of fatty liver was apparently suppressed.

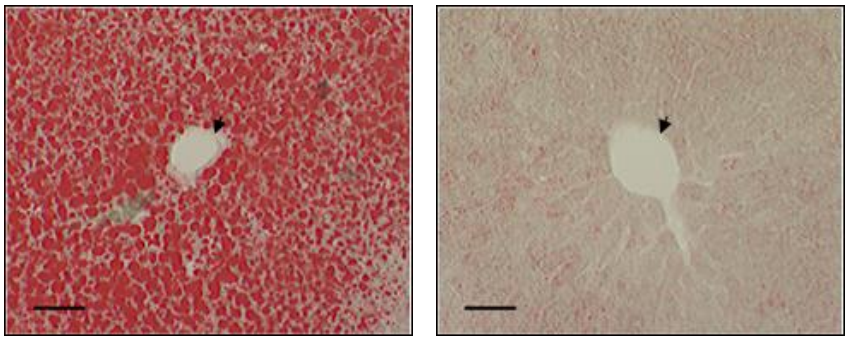

Figure1. Histlogical analysis of the liver (Oil Red-O staining)

The liver was extracted 14 days after commencing the treatment. Oil Red-O staining was performed in frozen liver sections to detect the presence of fat lipid droplets are stained red with Oil Red-O. The arrowhead shows the lumen of a blood vessel. These images are representative of observations made on 8 mice per groups. (Scale bar: 50 μm)

Table4. Results of hepatic histlogical analysis

	HFD								HFD+PFE							
	Animal no.								Animal no.							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Fatty liver	+++	+	++	+++	+++	+++	+	+++	-	-	-	-	-	-	-	-

-: no abnormality, ±: minor, +:slight, ++: moderate, +++: severe

Real-time quantitative RT-PCR

Table 5 shows the effect of PFE on mRNA expression in the liver, WAT, and BAT. The expression of hepatic genes involved in lipogenesis such as acetyl-CoA carboxylase (ACC) in the HFD+PFE group was significantly lower than that in the HFD group. For WAT, hormone-sensitive lipase (HSL) was significantly upregulated in epididymal adipose tissue in the HFD+PFE group. Similarly, uncoupling protein1 (UCP1) in BAT was significantly upregulated in the HFD+PFE group. The expression of genes related to beta-oxidation, such as Carnitine palmitoyltransferase1

(CPT1), Medium chain acyl CoA dehydrogenase (MCAD), and acyl-CoA oxidase (ACO), was not significantly different between the 2 groups, although their expressions were higher in the HFD+PFE group. These results suggest that PFE has anti-obesity effects in high-fat diet-induced obese mice through suppressing lipogenesis in the liver, stimulating lipolysis in WAT, and promoting thermogenesis in BAT.

Table5. Effect of PFE on mRNA levels in the liver, WAT, and BAT

	HFD	HFD+PFE	
Liver			
FAS	1.00 ± 0.28	0.58 ± 0.10	
ACC	1.00 ± 0.11	0.70 ± 0.06	*
CPT1	1.00 ± 0.14	1.21 ± 0.14	
MCAD	1.00 ± 0.23	1.51 ± 0.26	
ACO	1.00 ± 0.14	1.35 ± 0.20	
Epididymal adipose tissue			
HSL	1.00 ± 0.14	1.84 ± 0.15	**
Brown adipose tissue			
UCP1	1.00 ± 0.23	2.15 ± 0.38	*
PGC1a	1.00 ± 0.15	1.30 ± 0.08	

a) The data represent the mean ± SEM values (n = 8)

b) * and ** indicate significantly different at $p < 0.05$, $p < 0.01$, respectively.

Discussion

The Kudzu flower is a rich source of isoflavones [15], and soy isoflavones such as genistein and daidzein have been reported to exert anti-obesity effects [15, 16, 17]. Recently, Kim *et al.* reported that daidzein supplementation prevented non-alcoholic fatty liver disease in an animal study [18], and thus, it is believed that isoflavones are promising compounds for preventing obesity and fatty liver disease.

In this study, PFE supplementation significantly reduced body weight, body-weight gain, and WAT weight without affecting energy intake (i.e., food intake and fecal lipid content). It is known that obesity develops when energy intake exceeds energy expenditure. Therefore, PFE was believed to exert anti-obesity effects by increasing energy expenditure.

We previously reported that the isoflavone fraction from *Puerariae thomsonii* stimulated body weight loss in mice [18]. Soy isoflavone is reported to exert anti-obesity effects through suppressing lipogenesis in the liver by increasing protein kinase A activity [19] and promoting lipolysis in WAT by increasing cAMP levels [20, 21]. Moreover, tectoridin, an isoflavone characteristic of PFE, has been reported to modulate the expression of beta-oxidation genes such as MCAD and ACO in mice with ethanol-induced liver steatosis [22]. In this study, PFE suppressed the expression of ACC and FAS, which are rate-limiting enzymes in fatty-acid biosynthesis, in the liver. In addition, the expression of HSL, the predominant lipase effector of catecholamine-stimulated lipolysis, was also upregulated in WAT. Moreover, insignificant increases in the expression of beta-oxidation genes such as CPT1, MCAD, and ACO were observed (Table 5). These results are similar to the anti-obesity effects of isoflavones [19, 20, 21]; therefore, the active ingredient of PFE may be an isoflavone.

In addition, PFE supplementation significantly upregulated UCP1 expression in BAT. UCP1 is a key factor that determines the level of thermogenesis in BAT, and peroxisome proliferator-activated receptor gamma coactivator1a (PGC1a) is known to control UCP1 expression and mitochondriogenesis. A number of studies using mice revealed that UCP1 in BAT controls body fat levels by promoting energy expenditure [23, 24, 25]. Research about the effects of isoflavones on UCP1 expression in BAT is sparse; however, it is generally known that cAMP promotes UCP1 expression in BAT [26]. Therefore, PFE may promote UCP1 expression by increasing cAMP levels in BAT as observed in WAT. These results suggest that PFE affects energy expenditure; however, we have to validate this hypothesis by measuring oxygen consumption.

NASH is associated with progressive liver disease, fibrosis, and cirrhosis. Its pathogenesis is considered to include 2 steps. The first step is the development of hepatic steatosis due to the accumulation of free fatty acids in the liver, and the second step involves additional biochemical insults, including oxidative stress, the upregulation of inflammatory mediators, and dysregulated apoptosis [27, 28]. Currently, therapeutic options for NASH are limited to medications that reduce

the risk factors. Therefore, suppressing hepatic lipid accumulation, the first step of the pathogenesis of NASH, appears to be very important for preventing this hepatic disorder. In this study, PFE supplementation suppressed the development of fatty liver (Table 4). In addition, genes related to lipogenesis were significantly downregulated in the liver by PFE ingestion (Table 5). Thus, PFE might be expected to prevent NASH by suppressing hepatic lipid accumulation. In fact, GOT, GPT, and gamma-GTP expressions were significantly reduced by PFE ingestion in our preliminary clinical study [5]. This result supports the possibility that PFE supplementation provides the dual effects of preventing both obesity and hepatic disorders.

In the present study, the flower extract of *Puerariae thomsonii* have been demonstrated to possess anti-obesity and anti-fatty liver by in vivo assay. In addition, the flower extract of *Puerariae thomsonii* appears to exert these effects through suppressing lipogenesis in the liver and promoting lipolysis in white adipose tissue and thermogenesis in brown adipose tissue. In future research, we must clarify the detailed mechanism of PFE and its effects on energy expenditure.

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Section 3

Effect of *Pueraria* Flower Extract and Its Isoflavone-rich Fraction on Oxygen Consumption

Introduction

Puerariae flower extract (PFE) is a crude extract of the Kudzu flower (*Puerariae thomsonii*). It consists of approximately 20% isoflavones as the primary component. Kudzu is a leguminous plant found in Japan, China, and other areas, and its flower is a traditional Chinese medicine frequently used for counteracting symptoms associated with alcohol use, liver injury, and menopause. We previously reported preliminary findings demonstrating the anti-obesity effect of PFE in obese humans [1]. Another clinical study revealed that it reduces visceral fat area with no sexual dimorphism [2]. In addition, we previously reported that the active ingredient causing the anti-obesity effect of PFE is thought to be an isoflavone (See Chapter 2). We also reported that PFE exerts anti-obesity and anti-fatty liver effects in high-fat diet-fed mice. This occurs by upregulating the hormone-sensitive lipase in white adipose tissue (WAT) and the uncoupling protein 1 (UCP1) in brown adipose tissue (BAT) and through suppression of hepatic acetyl-CoA carboxylase (ACC) at the mRNA expression level [3].

BAT is responsible for non-shivering thermogenesis and diet-induced thermogenesis, which both regulate body temperature and weight [4, 5]. Uncoupling protein 1 plays an important role in BAT thermogenesis, and mouse studies have shown a link between UCP1 and obesity [6]. Studies using 18F-fluorodeoxyglucose positron-emission tomographic and computed tomographic scans have revealed that human adults have active BAT [7-10]. As previously described, PFE supplementation significantly upregulates UCP1 mRNA expression in BAT; therefore, PFE is expected to also increase energy expenditure.

Here, we conducted an animal study to investigate the effects of PFE and the isoflavone-rich fraction (ISOF) on oxygen consumption and UCP1 protein expression levels in BAT.

Materials and Methods

Experimental materials

Puerariae flower extract was purchased from Ohta's Isan Co. Ltd. (Ushiku city, Japan). This compound contains 7 isoflavones: tectoridin (4.70%), tectorigenin 7-*O*-xylosylglucoside (8.37%), 6-hydroxygenistein-6,7-diglucoside (3.38%), glycitin (0.17%), tectorigenin (0.83%), glycitein (0.10%), and genistein (0.06%). All isoflavone standard preparations we used were purchased from either Nagara Science Co., Ltd. (Gifu, Japan) or Tokiwa Phytochemical Co., Ltd. (Chiba, Japan)

Fractionation

The extract was dissolved in 20% MeOH and then sequentially eluted with 20%, 40%, 60%, and finally 100% MeOH for column chromatography (Cosmosil 75C18-OPN, Nacalai Tesque Inc., Kyoto, Japan). The fraction obtained from 20% MeOH was considered to be fraction 1. Next, silica gel column chromatography (solvent A, 1:1:40 [v/v) MeOH:HCOOH:$CHCl_3$; solvent B, 10:1 [v/v) MeOH:HCOOH) was performed using the 60% MeOH fraction. The solvent-A-eluted fraction and the 40% MeOH fraction were then mixed to obtain the ISOF. Fraction 2 was obtained by mixing the solvent-B-eluted fraction with the 100% MeOH fraction (Fig. 1). The fractionation yields, calculated as the dry weight of the fraction, were 27.1% for ISOF, 67.4% for fraction 1, and 3.9% for fraction 2

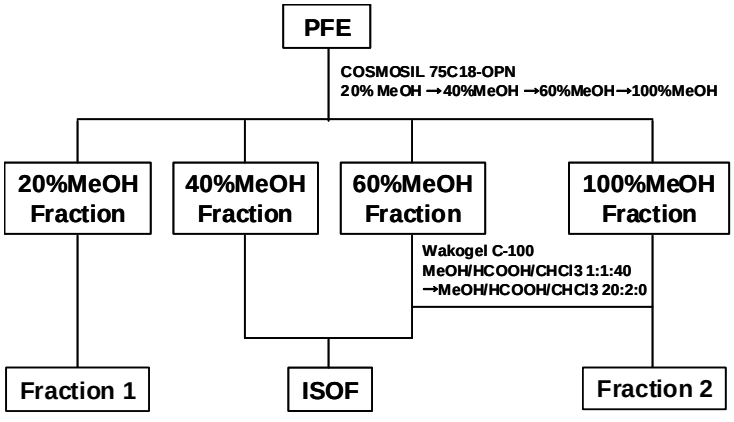

Figure1. Fractionation flow of ISOF prepared from PFE

Quantitative isoflavone estimation

The dietary soy isoflavone-aglycone testing method (See Food Safety Notification No. 0823001, August 23, 2006 for guidelines regarding handling of specified health food including soy isoflavones) was modified and implemented to quantify the amount of isoflavones present in PFE and each of the previously described fractions. Each fraction and PFE were dissolved in 50% EtOH and analyzed with high-performance liquid chromatography using a 4.6 × 250 mm YMC-pack ODS-AM-303 column, with UV detection at 264 nm. The flow rate was 1.0 mL/min. Solvent A was CH_3CN:H_2O:CH_3COOH at a ratio of 15:85:0.1 (v/v), and solvent B was CH_3CN:H_2O:CH_3COOH at a ratio of 35:65:0.1. Quantitative isoflavone results are shown in Table 1.

Table1. Results of quantitative isoflavone estimation in each fraction

(g/100g)

Constituent name	PFE	ISOF	Fraction1	Fraction2
6-Hydroxygenistein 6,7-di-O-glucoside	3.38	12.14	N.D.	N.D.
Glycitin	0.17	0.66	N.D.	N.D.
Tectorigenin 7-O-xylosylglucoside	8.37	30.35	N.D.	N.D.
Genistin	0.27	0.95	N.D.	N.D.
Tectoridin	4.70	16.30	N.D.	1.19
Glycitein	0.10	0.27	N.D.	N.D.
Genistein	0.06	0.16	N.D.	N.D.
Tectorigenin	0.83	2.30	N.D.	N.D.
Total	17.87	63.11	N.D.	1.19

Experimental animals and diet

All animal procedures were performed in accordance with the Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animal Science and were approved by the Ethical Committee of Toyo Shinyaku Co., Ltd. Male C57BL/6J mice were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan) at the age of 6 weeks. At 7 weeks, the mice were divided into 3 groups: (1) high-fat diet (HFD), (2) high-fat diet and given

5% PFE (HFD + PFE), and (3) high-fat diet and given PFE ISOF (HFD + ISOF). All groups were fed for 42 days. For the HFD + ISOF group, 1.355% ISOF from the fractionation yield was used. The animals were kept in an air-conditioned environment with a 12-h light cycle (lights on from 0800–2000). Mice were fed ad libitum during preparatory breeding and were on a controlled feeding regimen during the testing period. During the study, the animals were weighed every 4 days. Food intake was determined every day by subtracting the food remaining in the feed container from the total amount given the day before. The feed composition is shown in Table 2.

Measurement of Oxygen Consumption and Respiratory Quotient

We measured oxygen consumption and respiratory quotient (RQ) by using an expired-gas analyzer (Oxymax, Columbus Instruments, OH, USA) at days 35–39

Table2. Feed composition

(g/100g)

	HFD	HFD+PFE	HFD+ISOF
Casein	20.0	20.0	20.0
α-potato starch	28.2	23.2	26.845
Sucrose	13.0	13.0	13.0
Corn oil	20.0	20.0	20.0
Rard	10.0	10.0	10.0
Cellulose	4.0	4.0	4.0
Mineral Mix (AIN-76)	3.5	3.5	3.5
Vitamin Mix (AIN-76)	1.0	1.0	1.0
DL-Methionine	0.3	0.3	0.3
PFE	0.0	5.0	0.0
ISOF	0.0	0.0	1.355
Total	100.0	100.0	100.0

BAT Immunostaining and UCP1-Positive Area Ratio Measurement

The excised BAT was fixed in 10% formalin and sliced after paraffin embedding. The tissues were submitted for UCP1 immunostaining after deparaffinization. They were then processed with a polyclonal anti-UCP1 rabbit antibody (Abcam, Cambridge, UK) according to the

avidin-biotin-peroxidase complex method. Based on the method of Cinti *et al.* [11], the UCP1-positive area was calculated as follows: digital images were captured using a camera installed in the optical microscope (IX-70, Olympus Corporation, Tokyo, Japan), and the UCP1-positive area was measured using image analysis software (WinROOF V5.6, Mitani Corporation, Tokyo, Japan). The UCP1-positive area in the digital image was divided by the total tissue area to calculate the UCP1-positive area ratio

Statistical Analysis

Data were expressed as the mean ± SEM. For comparisons between groups, Fisher's protected least significance difference (PLSD) test was used. All statistical analyses were performed using Statview (version 5.0, SAS Institute Japan Ltd., Tokyo, Japan), and significance was set at $p < 0.05$.

Results

Oxygen Consumption and Respiratory Quotient

Figure 2 shows oxygen consumption and RQ on days 35–39 after the start of the experiment. As compared to the HFD group, the HFD + PFE and HFD + ISOF groups had significantly higher oxygen consumption but no significant differences in RQ. These results suggest that PFE and ISOF increase energy consumption without affecting RQ.

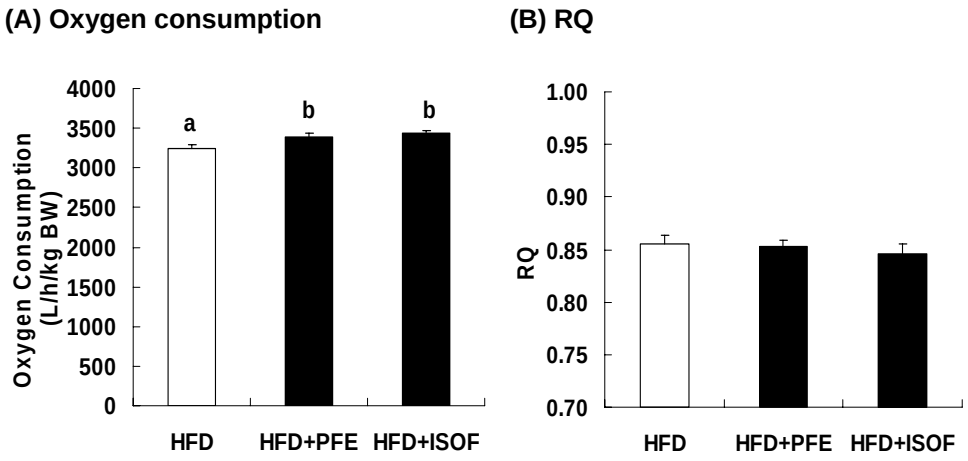

Figure2. Effects of PFE and ISOF on oxygen consumption and RQ

The data represent the mean ± SEM values (n = 7-8). Different symbols represent $p<0.05$ as compared with the other groups

BAT Immunostaining and UCP1-Positive Area Ratio

The HFD + PFE and HFD + ISOF groups had significantly higher UCP1-positive area ratios than the HFD group (Fig. 3). The HFD + PFE and HFD + ISOF groups also showed an apparent inhibition to fat accumulation in BAT. The BAT weight was significantly lower in both the HFD + PFE and HFD + ISOF groups compared to the HFD group (See Chapter 2), likely due to reduced fat accumulation.

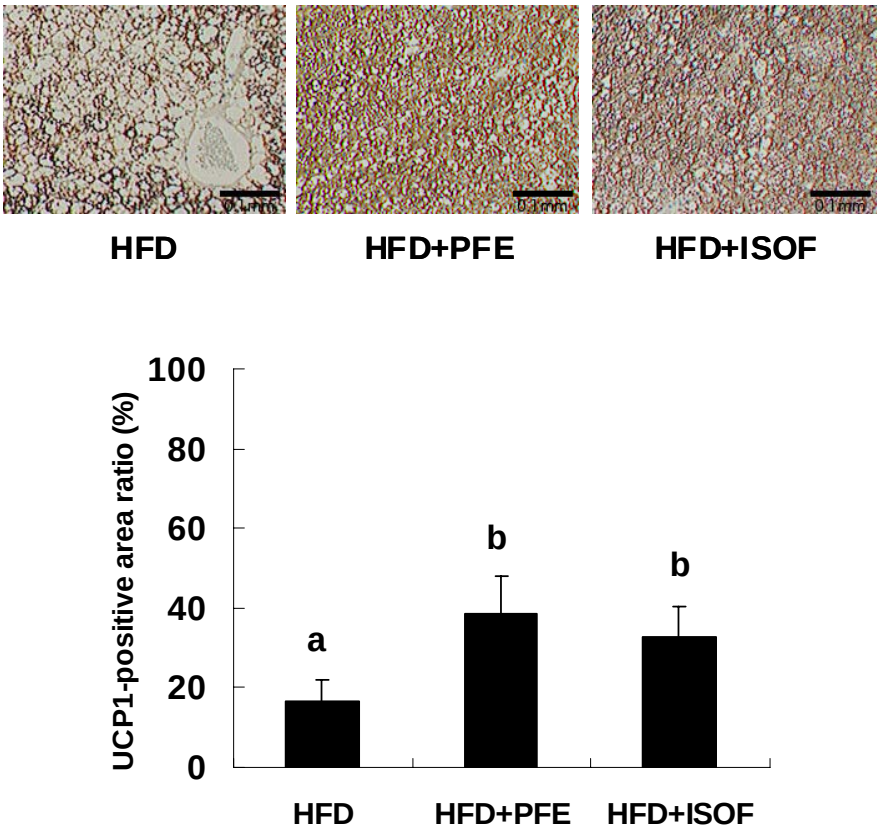

Figure3. UCP1 immunohistochemical staining and UCP1-positive area ratio in BAT

The data represent the mean ± SEM values (n = 6-8). Different symbols represent $p<0.05$ as compared with the other groups

Discussion

Uncoupling protein 1 is a key factor that determines the level of thermogenesis in BAT, and a number of mice studies have revealed that it controls body fat levels by promoting energy expenditure [6, 12, 13]. Brown adipose tissue promotes the hydrolysis of stored triglycerides by endogenous lipases, leading to the mobilization of fatty acid as fuel for thermogenesis. Cyclic-AMP promotes lipolysis and UCP1 expression, which are important factors for BAT thermogenesis, and activates cAMP-dependent protein kinase (PKA), which promotes lipolysis of triglycerides. Moreover, the cAMP response element binding protein (CREBP) increases UCP1 expression [14].

Flavonoids, such as genistein and quercetin, increase cAMP in adipocytes [15, 16], but there have been no in vivo studies on the effects of soy isoflavones on oxygen consumption. In the current study, PFE and ISOF significantly increased UCP1 expression in BAT as well as oxygen consumption. Supplementation with PFE did not significantly upregulate gene expression related to beta oxidation [3]; therefore, ISOF-induced increases in energy expenditure may be largely due to upregulation of BAT UCP1 expression. These findings provide valuable information regarding the anti-obesity effects of isoflavones.

The oxygen consumption of the HFD + PFE and HFD + ISOF groups were significantly higher than the HFD group, 4.7% and 6.0% respectively (Fig 1). C57BL/6J mice reportedly use 6–7 kcal per day [17]; therefore, PFE and ISOF supplementation seem to increase energy expenditure ~15 kcal over 42 days. Since adipose tissue contains 7 kcal/g, PFE and ISOF supplementation led to ~2 g of WAT reduction in these mice. In this study, the WAT weights of the HFD + PFE and HFD + ISOF groups (-2.4 g and -3.1 g, respectively) were significantly lower than that of the HFD group (See Chapter 2). These results suggest that PFE and ISOF-induced increases in energy expenditure produce significant WAT reductions.

In conclusion, anti-obesity action of PFE and its isoflavone rich fraction are likely at least partly responsible for increasing energy expenditure by upregulation of BAT UCP1 expression.

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Summary

Chapter 1

In this section, we conducted an animal study to investigate the effects of *Puerariae* flos extract (PFE) and the isoflavone-rich fraction (ISOF) of PFE on adipose tissue weight and hepatic triglyceride levels. In addition, we analyzed fecal lipid to investigate PFE affects lipid absorption or not. As a result, final body weight, body weight gain, white and brown adipose tissue weights, and hepatic triglyceride level were significantly lower in the HFD + PFE and in the HFD+ISOF groups compared to the HFD group. On the other hand, there were no significant differences between the HFD + PFE and HFD + ISOF groups in final body weight, weight gain, or white adipose tissue weight. In addition, no significant differences were observed in food intake or fecal lipid levels between the HFD + PFE and HFD + ISOF groups compared to the HFD. These results suggest PFE exerts anti-obesity and anti-fatty liver effects, and that the active ingredient causing the anti-obesity effect of PFE is thought to be an isoflavone.

Chapter 2

Section 1

This research is preliminary study to examine whether *Puerariae* flos extract (PFE) makes any action to body fat of man or not. We conducted the double-blind placebo controlled study on eighty mildly obese subjects for 8 weeks. The subjects were randomly divided into 4 groups—I, II, III, and IV. Subjects in groups I, II, and III consumed test food containing 100, 200, and 300 mg PFE, respectively, while those in group IV were given placebo food for 8 weeks. All subjects were instructed to restrict their total-energy intake to within 2650 kcal/day in the case of males and 2300 kcal/day in the case of females during the test period. Haematological and biochemical markers of blood, urinary markers, and physical markers were examined at 0, 4, and 8 weeks during the test period. As a result, no adverse effects were noted in any of the groups. For physical examinations,

we analysed only males whose initial body mass index (BMI) values were over 24. BMI value and body weights of the subjects in group III were significantly reduced, and total fat area and subcutaneous fat area of the subjects in groups II and III were significantly reduced over the 8-week test period. Moreover, as a result of comparison among groups by unpaired t-test, significant difference appeared between group III and group IV in BMI. Total fat area in group III tend to decrease compared with group IV. By these results, the reduction of BMI in group III may reflect fat area reduction. To confirm the anti-obesity effect of PFE, we need to research particularly by extensive studies hereafter.

Section 2

In this section, we have reported a confirmatory clinical trial to investigate the effects of the *Pueraria thomsonii* flower extract (PFE) on obesity by using obese Japanese males and females (BMI $\geq$ 25 kg/m^2). Eighty-one obese subjects were randomly divided into three groups and consumed test food containing 300 mg of PFE, 200 mg of PFE, and a placebo over 12 weeks. The results indicate that PFE intake reduces BMI and decreases, the visceral fat area, but not the subcutaneous fat area. In addition, the decrease in visceral fat area showed no sexual dimorphism. Consequently, we propose that PFE intake expresses its BMI reduction effects *via* a decrease in visceral fat area.

Chapter 3

Section 1

Phytoestrogens have recently received great attention because of their beneficial effects, including the prevention of atherosclerosis, and bone density loss. In particular, recent studies revealed that ERs affect adipose tissue weight. In this section, we conducted ER-binding assays to investigate the relative estrogenic activities of 6-hydroxygenistein and tectorigenin, the aglycone forms of PFE's major isoflavones as well as uterotrophic bioassays to investigate the estrogenic

effects of PFE *in vivo*. The ER-binding assays revealed that the ER-binding affinities of 6-hydroxygenistein and tectorigenin were approximately 0.01-0.04 that of genistein. The results of the uterotrophic bioassays demonstrate that PFE cannot exert estrogenic activity, even at a dose of 1000 mg/kg/day, which is the limit dose designated in the current draft protocol by the OECD. These results suggest that the mechanism of anti-obesity effect of PFE is not mediated by ERs.

Section 2

In this section, we conducted an animal study to investigate the effect of *Pueraria thomsonii* flower extract (PFE) on gene expression profiles.

Male C57BL/6J mice were fed a high-fat diet (HFD) or an HFD supplemented with 5% PFE for 14 days. PFE supplementation significantly reduced body weight and white adipose tissue (WAT) weight. Moreover, in the histological analysis, PFE supplementation improved fatty liver. Hepatic reverse transcription–polymerase chain reaction revealed that PFE supplementation downregulated acetyl-CoA carboxylase expression. For adipose tissue, the expressions of hormone-sensitive lipase in WAT and uncoupling protein 1 in brown adipose tissue (BAT) were significantly upregulated. These results suggest that PFE exerts anti-obesity and anti-fatty liver effects in high-fat diet-induced obese mice through suppressing lipogenesis in the liver, stimulating lipolysis in WAT, and promoting thermogenesis in BAT.

Section 3

As previously described, PFE supplementation significantly upregulates UCP1 mRNA expression in BAT; therefore, PFE is expected to also increase energy expenditure. Here, we conducted an animal study to investigate the effects of PFE and the isoflavone-rich fraction (ISOF) on oxygen consumption and UCP1 protein expression levels in BAT. PFE and ISOF significantly increased UCP1 expression in BAT as well as oxygen consumption. As a result, supplementation with PFE did not significantly upregulate gene expression related to beta oxidation (See chapter 3

Section 2); therefore, ISOF-induced increases in energy expenditure may be largely due to upregulation of BAT UCP1 expression. These results suggest that anti-obesity action of PFE and its isoflavone rich fraction are likely at least partly responsible for increasing energy expenditure by upregulation of BAT UCP1 expression.

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